EVIDENCE APPENDIX
EXHIBIT 1

Declaration of Mahendra S. Rao, M.D., Ph.D. under 37 C.F.R. § 1.132

PATENT
Docket No.: 19603/4230 (CRF D-2238B)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: R. Hutson
Serial No.	:	09/282,239	
Cnfrm. No.	:	8339	Art Unit: 1652
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

DECLARATION OF MAHENDRA S. RAO, M.D., PH.D. UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, MAHENDRA S. RAO, M.D., Ph.D., pursuant to 37 C.F.R. § 1.132, declare:

1. I received an M.D. (MBBS) degree in Medicine from Bombay University, Bombay India and a Ph.D. degree in Medicine from California Institute of Technology in Pasadena, California.

2. I am a Section Chief for the Stem Cell Unit at the Laboratory of Neuroscience, at NIA (National Institute on Aging), Triad Technology Center, 333 Cassell Drive, Baltimore, MD 21224; an Associate Professor of Neurosciences at Johns Hopkins University School of Medicine, Baltimore, MD 21224; and an Associate Professor at NCBS, Bangalore, India.

3. I am a founder of and shareholder in Q Therapeutics, Inc., 615 Arapeen Drive, Suite 102, Salt Lake City, Utah 84108, which I understand has an exclusive license under the present patent application.

4. I am familiar with the subject matter of the present patent application which I understand is directed to an enriched or purified preparation of human mitotic oligodendrocyte progenitor cells where the cyclic nucleotide phosphodiesterase 2 promoter (i.e. CNP2") is transcriptionally active in all cells of the enriched or purified preparation.

5. I am a co-inventor of U.S. Patent No. 6,361,996 ("'996 Patent"), which I understand has been used as a basis for rejecting claims in the above application. I present this declaration to demonstrate why the subject matter of the '996 Patent is very different from that of the present patent application.

6. The '996 Patent discloses multipotential neuroepithelial stem cells and lineage-restricted astrocyte/oligodendrocyte precursor cells. The astrocyte/oligodendrocyte precursor cells are derived from neuroepithelial stem cells, are capable of self-renewal, and can differentiate into astrocytes and oligodendrocytes but not neurons. The '996 Patent characterizes these cells as "multipotential intermediate precursor cells restricted to glial lineages" (emphasis added)(column 23, lines 1-5). Similarly, my paper Rao, et. al., "Glial-Restricted Precursors are Derived From Multipotential Neuroepithelial Stem Cells," *Devel. Biol.* 188: 48-63 (1997) clearly demonstrates that such A2B5+/NCAM cells are capable of generating both astrocytes and oligodendrocytes and do not appear committed to the oligodendrocyte lineage. The '996 Patent's astrocyte/oligodendrocyte precursor cells are in a less differentiated state than the oligodendrocyte progenitor cells of the present patent application and, therefore, are very different from the cells described in this present application.

7. Differences in the method, time of isolation, and propagation should also be noted. The cells in the present application were derived from the adult brain using a promoter reporter based strategy where the CNP2 promoter directed expression of green fluorescent protein. On the other hand, the astrocyte/oligodendrocyte precursor cells of the '996 Patent were derived from fetal and neonatal tissue using cell surface antigen expression and fluorescence based antibody capture. No strategy of using CNP2 (a cytoplasmic marker) expression, a CNP2 promoter, or a related promoter reporter strategy is described in the '996 Patent.

8. The '996 Patent is directed to the enrichment of glial progenitor cells from newborn rat brain. Newborns have an abundant population of still-developing oligodendrocyte progenitor cells that may constitute a significant fraction of all of the cells in neonatal brain tissue. Yakovlev, et. al., "A Stochastic Model of Brain Cell Differentiation in Tissue Culture," *J Math Biol.*, 37(1):49-60 (1998)(Appendix 1); Bogler et. al., "Measurement of Time in Oligodendrocyte-type-2

Astrocyte (O-2A) Progenitors is a Cellular Process Distinct from Differentiation or Division," *Dev Biol.*, 162(2):525-38 (1994)(Appendix 2); Raff et. al., "Platelet-derived Growth Factor From Astrocytes Drives the Clock That Times Oligodendrocyte Development in Culture." *Nature* 333(6173):562-65 (1988)(Appendix 3) describe cell cycle changes as glial progenitor cells mature. They showed that adult cells differ in their cell cycle time and the number of divisions before they will become postmitotic. The present patent application discloses this for adult human-derived cells. In addition, adult-derived human oligodendrocyte progenitor cells differentiate as oligodendrocytes and produce myelin much more quickly than do fetal or neonatal oligodendrocyte progenitor cells. In particular, as recently reported in Nunes et al., "Identification and Isolation of Multipotent Neural Progenitor Cells from the Subcortical White Matter of the Adult Human Brain," *Nature Medicine* 9:239-247 (2003) (Appendix 4) and Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cell Isolates Myelinate the Congenitally Dysmyelinated Brain," *Nature Medicine* 10:93-97 (2004) (Appendix 5), adult-derived oligodendrocyte progenitor cells not only myelinate much more rapidly than do fetal oligodendrocyte progenitors, but they do so more efficiently, with a higher proportion exhibiting effective myelin production, and myelinating a greater number of neuronal axons per donor cell than their fetal-derived counterparts. Adult cells are thus fundamentally more biased towards generating oligodendrocytes, towards maturing to express myelin proteins, and towards myelinating host axons. Moreover, adult cells execute all of these functions, and achieve each of these cellular milestones, much more quickly than fetal cells. As a result, they lend themselves to a very different set of potential clinical targets than fetal or neonatal-derived progenitors, as recently reported in Roy et al., "Progenitor Cells of the Adult Human Subcortical White Matter In: *Myelin Biology and Disorders*, vol. 1. R. Lazzarini, ed. Elsevier:Amsterdam, pp. 259-287 (2004) (Appendix 6). The adult oligodendrocyte progenitor cells of the present application are thus fundamentally different from the fetal or neonatal-derived astrocyte/oligodendrocyte precursor cells of the '996 Patent.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 10/14/04

Mahendra S. Rao MD., Ph.D.

EVIDENCE APPENDIX
EXHIBIT 2

Second Declaration of Mahendra S. Rao, M.D., Ph.D. under 37 C.F.R. § 1.132

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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SECOND DECLARATION OF MAHENDRA S. RAO, M.D., PH.D. UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
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7. As shown in Figures 1-2 of the '996 Patent, the astrocyte/oligodendrocyte precursor cells 14 and 54, respectively, differentiate directly into two cell types – i.e. of astrocytes and oligodendrocytes. We also know from clonal analysis that there is a homogeneous population of astrocyte/oligodendrocyte precursor cells in which individual cells generate oligodendrocytes and two kinds of astrocytes by the process described in the '996 Patent. It is important to note that multiple pathways to generate post-mitotic, mature oligodendrocytes, have been described. Anderson and colleagues have shown that an oligodendrocyte/motorneuron precursor exists that does not make astrocytes (Zhou et al., "The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification," *Cell* 109:61-73 (2002) (attached hereto as Exhibit 1)). Other investigators have shown distinct sites of origin of oligodendrocytes and astrocytes presumably from separate precursors (Vallstedt et al., "Multiple Dorsoventral Origins of Oligodendrocyte Generation in the Spinal Cord and Hindbrain." *Neuron* 45:55-67 (2005) (attached hereto as Exhibit 2) and Cai et al., "Generation of Oligodendrocyte Precursor Cells from Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling," *Neuron* 45:41-53 (2005) (attached hereto as Exhibit 3)). Yet other investigators have shown that different kinds of oligodendrocyte progenitors exist (Pringle et al., "*Fgfr3* Expression by Astrocytes and Their

Precursors: Evidence that Astrocytes and Oligodendrocytes Originate in Distinct Neuroepithelial Domains," *Development* 130:93-102 (2003) (attached hereto as Exhibit 4)). We are not aware of any evidence that the astrocyte/oligodendrocyte precursor cells of the '996 Patent generated mature oligodendrocytes by way of an intermediate oligodendrocyte-specific precursor. Indeed, Gregori et al., "The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal-Ventral Differences in GRP Cell Function," *J. Neurosci.* 22(1):248-256 (2002) (attached hereto as Exhibit 5) have suggested that the '996 patent describes a glial progenitor that gives rise to a more restricted astrocyte/oligodendrocyte precursor that still directly makes predominantly astrocytes and a small minority of oligodendrocytes. Thus, cells in the '996 Patent's pathway to oligodendrocyte production are bi-potential astrocyte/oligodendrocyte progenitor cells that have strong astrocytic bias. These cell types are very different from the oligodendrocyte-specified progenitor cells of the present application.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 05/10/05

Mahendra S. Rao MD., Ph.D.

EVIDENCE APPENDIX
EXHIBIT 3

Gregori et al., "The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal-Ventral Differences In GRP Cell Function," *J. Neurosci.* 22(1):248-256 (2002)

The Journal of Neuroscience, January 1, 2002, 22(1):248–256

The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal–Ventral Differences in GRP Cell Function

Ninel Gregori,[1]* Christoph Proschel,[2]* Mark Noble,[2] and Margot Mayer-Proschel[2]

[1]*University of Utah School of Medicine, Salt Lake City, Utah 84132, and* [2]*Center for Cancer Biology, University of Rochester Medical Center, Rochester, New York 14642*

We have found that the tripotential glial-restricted precursor (GRP) cell of the embryonic rat spinal cord can give rise *in vitro* to bipotential cells that express defining characteristics of oligodendrocyte-type-2 astrocyte progenitor cells (O2A/OPCs). Generation of O2A/OPCs is regulated by environmental signals and is promoted by platelet-derived growth factor (PDGF), thyroid hormone (TH) and astrocyte-conditioned medium. In contrast to multiple observations indicating that oligodendrocyte precursor cells in the embryonic day 14 (E14) spinal cord are ventrally restricted, GRP cells are already present in both the dorsal and ventral spinal cord at E13.5. Ventral-derived GRP cells, however, were more likely to generate O2A/OPCs and/or oligodendrocytes than were their dorsal counterparts when exposed to TH, PDGF, or even bone morphogenetic protein-4. The simplest explanation of our results is that oligodendrocyte generation occurs as a result of generation of GRP cells from totipotent neuroepithelial stem cells, of O2A/OPCs from GRP cells and, finally, of oligodendrocytes from O2A/OPCs. In this respect, the responsiveness of GRP cells to modulators of this process may represent a central control point in the initiation of this critical developmental sequence. Our findings provide an integration between the earliest known glial precursors and the well-studied O2A/OPCs while opening up new questions concerning the intricate spatial and temporal regulation of precursor cell differentiation in the CNS.

Key words: glial-restricted precursor cell; GRP cell; oligodendrocyte; O2A progenitor cell; OPCs; spinal cord development; ventral origin; neuroepithelial stem cells

Understanding how the differentiated cell types of the body are generated is a central challenge in developmental biology. Multiple components contribute to this process, including signaling molecules and transcription factors that cause precursor cells to progress along different developmental pathways. Central to understanding cell generation, however, is identification of the precursor cell from which a given cell type arises, for it is the specific precursor cell that represents the actual target for exogenous influences.

The creation of specific precursor cells and differentiated cell types proceeds through a sequence of lineage restrictions but also may involve a phenomenon of lineage convergence. Through lineage restriction, the totipotent stem cells of the earliest embryo generate progeny that are more restricted in the range of cell types they generate. For example, totipotent embryonic stem cells give rise to tissue-specific stem cells. Tissue-specific stem cells proceed to produce differentiated cell types via intermediate lineage-restricted precursor cells. These lineage-restricted precursor cells ultimately generate a subset of the differentiated cell types in a particular tissue. Lineage restriction is complemented in development by the process of lineage convergence, by which different lineages give rise to the same cell type. One example of such convergence is seen in the formation of cartilage from both mesenchymal and cranial neural crest lineage (Baroffio et al., 1991).

Studies on CNS development are revealing a rich diversity of precursor cells that can give rise to the same cell type, particularly with respect to glial development. For example, it is well established that oligodendrocytes can be generated from oligodendrocyte-type-2 astrocyte progenitor cells (Raff et al., 1983; Skoff and Knapp, 1991), which also are referred to as oligodendrocyte precursor cells (Raff et al., 1983; Skoff and Knapp, 1991) and abbreviated here as O2A/OPCs. More recent studies on embryonic rat spinal cord have led to the isolation of a new and distinct population, called tripotential glial-restricted precursor (GRP) cells, that also can generate oligodendrocytes *in vitro* and *in vivo* (Rao et al., 1998; Herrera et al., 2001). GRP cells and O2A/OPCs differ in several characteristics. For example, GRP cells and O2A/OPCs differ in their responses to mitogens, survival factors, and inducers of differentiation (Rao et al., 1998). GRP cells and O2A/OPCs also express distinct differentiation potentials *in vitro*: GRP cells are able to generate oligodendrocytes and two distinct astrocyte populations, whereas O2A/OPCs can generate oligodendrocytes and only one kind of astrocyte. Moreover, GRP cells readily generate astrocytes when transplanted into the neonatal or adult brain (Herrera et al., 2001), a cell type not generated from primary O2A/OPCs, after transplantation into the normal CNS (Espinosa de los Monteros et al., 1993).

Several critical questions arise from the fact that it now is possible to isolate two distinct precursor cell populations (i.e., GRP cells and O2A/OPCs) from the developing animal, each of

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*N.G. and C.P. contributed equally to this manuscript.

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Table 1. Differentiation potential of GRP-derived O4$^+$ and O4$^-$ cells

Culture condition	O4$^+$ cells		O4$^-$ cells	
	PDGF/TH	10% FCS	PDGF/TH	10% FCS
	Number of clones that contain specific cell types/number of all scored clones			
Type-2 astrocytes only	0/15	21/22	0/29	1/21
Type-1 astrocytes only	0/15	1/22	0/29	3/21
Type-1 and type-2 astrocytes	0/15	0/22	0/29	17/21
Oligodendrocytes and progenitors	15/15	0/22	29/29	0/21

Individual GRP cell-derived O4$^+$ and O4$^-$ cells were expanded to a clonal size of 5–10 cells before being exposed to 10% FCS to induce astrocytic differentiation or exposed to PDGF and TH to promote oligodendrocyte differentiation. After 5 or 10 d, respectively, clones were stained with anti-GFAP, A2B5, and anti-GalC antibodies. In serum-containing medium, all but one of the clones derived from O4$^+$ cells contained only astrocytes with the antigenic phenotype of type-2 astrocytes (i.e., GFAP$^+$ and A2B5$^+$). In contrast, clones generated from O4$^-$ cells contained a mixture of type-1 (i.e., GFAP$^+$ A2B5$^-$) and type-2 astrocytes. Although some clones derived from O4$^+$ or O4$^-$ cells contained one or two progenitor cells, none of the clones contained oligodendrocytes at that time point. All clones exposed to PDGF and TH, regardless of being derived from O4$^+$ or O4$^-$ cells, contained A2B5$^+$ progenitor cells and GalC$^+$ oligodendrocytes. None of these clones contained astrocytes.

which can generate oligodendrocytes. Is the relationship between these two populations one of lineage restriction or lineage convergence? If GRP cells and O2A/OPCs are related, what signals promote the generation of one from the other and how can the existence of both populations be integrated with existing studies on the generation of oligodendrocytes during spinal cord development?

MATERIALS AND METHODS

Cell culture. A2B5$^+$ GRP cells were isolated from embryonic day 13.5 (E13.5) Sprague Dawley rat spinal cords by positive selection on immunopanning dishes coated with A2B5 antibody (Rao et al., 1998). GRP cells were then grown in the presence of 10 ng/ml basic FGF (bFGF) and indicated supplements for various time points on fibronectin/laminin-coated coverslips at 3000 cells/well for mass culture experiments or on coated grid dishes for clonal analysis. Cultures were fed every other day with the factors indicated. At the end of the experiment, cells were stained with O4 (Sommer and Schachner, 1981) or A2B5 antibodies to detect precursor cells, anti-galactocerebroside (GalC) antibody (Gard and Pfeiffer, 1990; Gard et al., 1995) to identify oligodendrocytes, and anti-GFAP antiserum to identify astrocytes (Norton and Farooq, 1993; Morita et al., 1997; Gomes et al., 1999) followed by appropriate fluorochrome-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL). The number of cells of each type relevant to each experiment was calculated, as was the total cell number. As originally defined, GFAP$^+$ cells were scored as type-2 astrocytes if they were stellate and A2B5$^+$ and as type-1 astrocytes if they were fibroblast-like in morphology and were A2B5$^-$.

Rationale for use of the O4 antibody in analyzing generation of O2A/OPCs from GRP cells. To determine whether one cell type gives rise to another, it is useful to identify a marker that is expressed by one cell type but not by the other. This is particularly problematic for analysis of GRP cells and O2A/OPCs. Freshly isolated GRP and O2A/OPCs both label with the A2B5 monoclonal antibody. We have shown previously that GRP cells can express receptors for platelet-derived growth factor (PDGF) without losing their tripotentiality (Rao et al., 1998). Our ongoing studies have revealed that tripotential GRP cells also label with anti-GD3 and anti-NG-2 antibodies (C. Pröschel, D. Gass, and M. Mayer-Pröschel, unpublished observations). Thus, none of these markers, which have been used by many others to study development of O2A/OPCs (Hart et al., 1989; Yim et al., 1995; Nishiyama et al., 1996), allow a distinction to be made between GRP cells and O2A/OPCs.

At this stage, the only remaining candidate marker for investigating whether GRP cells can generate O2A/OPCs is the O4 monoclonal antibody (Sommer and Schachner, 1981). This antibody can be used to define a secondary stage of O2A/OPC development, in which A2B5$^+$ O4$^-$ O2A/OPCs give rise to cells that are A2B5$^+$ and also O4$^+$. The great majority of O2A/OPCs isolated from the p7 optic nerve are O4$^+$ (M. Noble, unpublished observations), whereas GRP cells are O4$^-$ (Rao and Mayer-Pröschel, 1997; Rao et al., 1998). In addition, it has been shown that development of GalC$^+$ oligodendrocytes in the O2A/OPC lineage is preceded by the appearance of cells that are O4$^+$ but GalC$^-$ (Schachner et al., 1981; Sommer and Schachner, 1981; Bansal et al., 1989; Gard and Pfeiffer, 1990, 1993). Critically, O4$^+$ GalC$^-$ cells isolated from many regions of the postnatal CNS, including spinal cord, are bipotential cells capable of differentiating into both oligodendrocytes and type-2 astrocytes (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999). O4$^+$ GalC$^-$ cells also can be induced to proliferate *in vitro* and in this respect are not terminally differentiated (Small et al., 1987; Trotter et al., 1989; Gard and Pfeiffer, 1990; Reynolds and Wilkin, 1991; Warrington and Pfeiffer, 1992; Avossa and Pfeiffer, 1993; Barnett et al., 1993; Gard et al., 1995). Thus, although some authors have preferred to consider O4$^+$ GalC$^-$ cells (isolated from postnatal animals or derived from O2A/OPCs) as more committed "oligodendroblasts," the O4$^+$ GalC$^-$ cells studied thus far express those characteristics (in particular, bipotentiality *in vitro* and ability to divide) that are most important in defining a cell as being a bipotential O2A/OPC.

Clonal analysis of E13.5 GRP cell-derived O4$^-$ cells. We confirmed the differentiation potential of a cell by clonal differentiation analysis, as used in our previous studies on GRP cells (Rao and Mayer-Pröschel, 1997; Rao et al., 1998) and extensive studies on O2A/OPCs (Ibarrola et al., 1996; Smith et al., 2000); this is the only technique that allows the differentiation characteristics of individual precursor cells to be unambiguously ascertained. The basic strategy used to conduct such analyses in the present studies we as follows: GRP cells were isolated from E13.5 spinal cord as described previously and grown either for 24 hr or for 21 d in the presence of bFGF (10 ng/ml) before being exposed to the condition most effective at generating O4$^+$ GalC$^-$ cells (i.e., chemically defined medium supplemented with 10 ng/ml PDGF-A chain homodimer; Peprotech, Rocky Hill, NJ) and thyroid hormone (TH; Sigma, St. Louis, MO). It is critical to note that GRP cells grown for 24 hr in FGF do not express PDGF receptor-α (PDGFR-α), whereas long-term cultured GRP cells express this receptor. We have determined that when grown in the presence of FGF, GRP cells remain tripotential regardless of their PDGF receptor status (Rao et al., 1998). After periods of additional *in vitro* growth indicated in Results, cultures were labeled with both O4 and anti-GalC antibodies, followed by appropriate fluorescein- and rhodamine-conjugated secondary antibodies. Fluorescence-activated cell sorting was then used to obtain populations of O4$^+$ GalC$^-$ cells. O4$^-$ GalC$^-$ cells were plated at clonal density and single O4$^+$ GalC$^-$ cells were identified and circled. Cells were induced to divide for 5 d (in PDGF/bFGF at 10 ng/ml), and clones were switched to PDGF plus TH or 10% FCS when they reached a density of 5–10 cells. After 10 or 3 d, respectively, clones were stained with the A2B5, anti-GFAP, and anti-GalC antibodies. Control cells were switched to PDGF plus TH or 10% FCS without previous proliferation and stained after an additional 10 and 3 d, respectively. The results of our clonal analyses are shown in Tables 1 and 2.

Immunostaining of clones. Staining procedures were as described previously (Rao and Mayer-Pröschel, 1997). Briefly, the A2B5 and anti-GalC antibodies were grown as hybridoma supernatants (American Type Culture Collection, Manassas, VA) and used at a dilution of 1:2. The O4 hybridoma cell line was a generous gift from Ilse Sommer (University of Glasgow, Glasgow, UK), and its supernatant was also used at a 1:2 dilution. Anti-GFAP (polyclonal, rabbit anti-cow; purchased from Dako, Glostrup, Denmark) was used at a 1:100 dilution and applied overnight. All secondary antibodies [i.e., goat anti-mouse IgM-biotin, IgG3-tetramethylrhodamine B isothiocyanate, goat-anti-rabbit Ig (heavy and light chain)-FITC (Southern Biotechnology), and streptavidin (Molecular Probes, Eugene, OR)] were used at a 1:100 dilution. Anti-NG2

Table 2. NSC-derived A2B5$^+$ cells represent GRP cells

Antigenic phenotypes of cells within clones	Ventral E10.5 spinal cord-derived					Dorsal E10.5 spinal cord-derived				
	GFAP$^+$ A2B5$^+$ and GFAP$^+$ A2B5$^-$	GFAP$^+$ A2B5$^-$ only	GFAP$^+$ A2B5$^-$ only	A2B5$^+$ only	GalC$^+$	GFAP$^+$ A2B5$^+$ and GFAP$^+$ A2B5$^-$	GFAP$^+$ A2B5$^-$ only	GFAP$^+$ A2B5$^+$ only	A2B5$^+$ only	GalC$^+$
Percentage of clones (total number) after 7 d in 10% FCS/bFGF	70 (59)	0	0	30 (26)	0	83 (68)	0	0	17 (14)	0
Percentage of clones (total number) after 10 d in TH/bFGF	29 (33)	0	0	36 (40)	35 (39)	34 (23)	0	0	41 (29)	25 (18)

NSCs were isolated from dorsal and ventral regions of the E10.5 spinal cord and expanded in nondifferentiation conditions. After 3 d, dorsal and ventral cultures were allowed to differentiate, and the appearing A2B5$^+$ cells were harvested and replated at clonal density. Expanded clones were exposed either to 10% FCS or to TH to promote astrocytic and oligodendrocytic differentiation, respectively (both conditions also contained FGF). Both ventral- and dorsal-derived clones generated two types of astrocytes in the presence of 10% FCS and GalC$^+$ oligodendrocytes in the presence of TH. All clones that contained oligodendrocytes also contained A2B5$^+$ progenitor cells. We did not see any clones that contained only one type of astrocytes. The numbers shown refer to the number of clones containing different cell types but not the relative composition of the entire culture. For example, the great majority of astrocyte-containing clones in TH only had 1–5% GFAP$^+$ cells, suggesting that the presence of FGF allows some astrocyte differentiation to occur even when TH is present. Numbers in parentheses refer to the total number of clones, whereas numbers without parentheses indicate the percentage of clones in each category.

antiserum was a generous gift from Dr. W. Stallcup (Burnham Institute, La Jolla Cancer Research Center, CA) and was used at a 1:100 dilution.

RESULTS

Tripotential GRP cells, which are O4$^-$ cells, generate bipotential O4$^+$GalC$^-$ cells when grown in the presence of PDGF and thyroid hormone

The first question we addressed was whether tripotential GRP cells can generate cells with the antigenic and differentiation characteristics of bipotential O2A/OPCs. This question was investigated by a combined analysis of antigen expression and of differentiation potential at the clonal level. The requirement to use the O4 antibody (Sommer and Schachner, 1981) as a potential marker of O2A/OPCs is explained in Materials and Methods. Briefly, both GRP cells and O2A/OPCs label with the A2B5 antibody, the NG-2 antibody (Stallcup and Beasley, 1987), and the anti-GD3 antibody (Seyfried and Yu, 1985), and both populations can express PDGF receptors while maintaining their characteristic differentiation potential. Thus, of all of the markers that have been used to study the ancestors of oligodendrocytes, it was only the O4 antibody that remained potentially useful in this context. We designed experiments that would allow us to answer the following questions: (1) are there *in vitro* growth conditions that promote the generation of O4$^+$GalC$^-$ cells from O4$^-$ GRP cells, and (2) do GRP cell-derived O4$^+$GalC$^-$ cells still behave like tripotential GRP cells or do they now behave like bipotential O2A/OPCs?

We first examined the effects on GRP cells of a wide variety of conditions (see Materials and Methods) shown previously to induce generation of oligodendrocytes in cultures of O2A/OPCs. Although astrocyte-conditioned medium in combination with TH was the most effective condition for inducing the appearance of oligodendrocytes over a 3 d time period (data not shown), it was growth in the presence of FGF plus PDGF plus TH that was associated with the generation of the greatest proportion of O4$^+$GalC$^-$ cells.

In cultures of freshly isolated GRP cells that were grown for 24 hr in the presence of FGF and then additionally exposed to PDGF plus TH (with FGF still present), 78 ± 9% of the cells were O4$^+$GalC$^-$ after 3 d in culture. In addition, we noticed that 20 ± 5% of all cells were O4$^+$GalC$^-$ oligodendrocytes and a small percentage (2 ± 0.7%) of cells represented GFAP$^+$ astrocytes. We never observed the appearance of any cells that were GalC$^+$ but O4$^-$, consistent with previous observations that passage through an O4$^+$ stage is required before the expression of GalC immunoreactivity (Schachner et al., 1981; Sommer and Schachner, 1981; Bansal et al., 1989; Gard and Pfeiffer, 1990, 1993). GRP cell cultures that were grown in the presence of FGF alone contained no O4$^+$ cells, and previous studies have demonstrated that GRP cells expanded in this manner retain the ability to generate oligodendrocytes, type-1 astrocytes, and type-2 astrocytes.

Although previous studies have shown that O4$^+$GalC$^-$ cells isolated from postnatal animals or derived from bipotential O2A/OPCs are bipotential *in vitro* (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999), it cannot be assumed that such differentiation characteristics necessarily apply to O4$^+$GalC$^-$ cells derived from tripotential GRP cells. To determine the differentiation potential of GRP cell-derived O4$^+$GalC$^-$ cells, we cultured expanded GRP cells in the presence of FGF for several days, grew them in the additional presence of PDGF plus TH for 3 more days, purified the O4$^+$GalC$^-$ cells, and analyzed their differentiation potential in clonal cultures. Extending the previous expansion period in FGF in this manner resulted in a higher percentage of the cells in the culture remaining O4$^-$, thus allowing the study of this population also.

Cloned O4$^+$GalC$^-$ cells derived from GRP cells expressed the bipotential differentiation characteristics associated with O2A/OPCs. When grown in conditions that induced generation of astrocytes, O4$^+$GalC$^-$ cells derived from GRP cells exhibited the typical differentiation response of O2A/OPCs. In the presence of 10% FCS, the only astrocytes generated in 21 of 22 clones derived from O4$^+$GalC$^-$ cells were type-2 astrocytes (i.e., A2B5$^-$GFAP$^+$ stellate cells; Table 1 and Fig. 1*A*). Only one clone generated type-1-like astrocytes (i.e., A2B5$^-$GFAP$^+$ cells with a fibroblast-like morphology), a frequency low enough to be consistent with the possibility that this one clone had been mislabeled at the beginning of the experiment. This outcome was very different from that obtained with GRP cells themselves, clones of which generate a combination of type-1 and type-2 astrocytes in these conditions (Rao et al., 1998). Moreover, the O4$^+$GalC$^-$ cells that remained after the purification process were still tripotential, emphasizing that the acquisition of bipo-

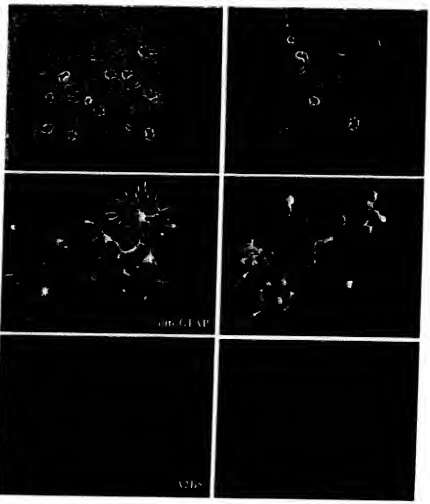

Figure 1. GRP-derived O4$^+$ cells are bipotential and represent O2A/OPC-like cells. Freshly isolated GRP cells were grown for 3 weeks in defined medium in the presence of bFGF and then switched to a medium supplemented with bFGF and TH; after 5 d, cultures were stained with the O4 antibody (see Materials and Methods). Cells were then dislodged from the surface and plated at clonal density in poly-L-lysine-coated dishes. Single O4$^+$ cells were circled. After 3 d in culture, cells were exposed to medium supplemented with bFGF and 10% FCS. (Parallel experiments using BMP6 instead of FCS yielded identical results.) After 5 d, clones were stained with A2B5 (rhodamine), anti-GFAP (fluorescein), and anti-GalC (coumarin) antibodies. The coumarin staining is not shown because none of the clones contained any GalC$^+$ oligodendrocytes in this condition. *A*, Clone derived from a single O4$^+$GalC$^-$ cell. The progeny from O4$^+$ founder cells consists exclusively of A2B5/GFAP double-positive cells, consistent with the antigenic phenotype of type-2 astrocytes. *B*, Clone derived from a single O4$^-$GalC$^-$ cell. The progeny from O4$^-$ founder cells consists of A2B5/GFAP double-positive type-2 astrocytes and A2B5$^-$GFAP$^+$ cells, representing type-1 astrocytes (indicated by *arrows*).

tentiality was a specific event and not merely associated with aging, even in conditions that promote the transition to a bipotential phenotype. When grown in the presence of 10% FCS, O4$^-$GalC$^-$ cells generated clones containing a mixture of type-1 and type-2 astrocytes (Table 1 and Fig. 1*B*), and thus behaved as GRP cells. In contrast to this difference with respect to astrocyte induction, both O4$^+$GalC$^-$ and O4$^-$GalC$^-$ cell-derived clones contained oligodendrocytes when grown in the presence of PDGF plus TH.

The simplest explanation of the data obtained in the above experiments is that GRP cells can generate O4$^+$GalC$^-$ cells that exhibit *in vitro* the defining bipotential differentiation restriction of O2A/OPCs. The results also indicate that generation of such bipotential cells is an environmentally regulated differentiation event, for which PDGF and TH represent potent inducing agents.

GRP cells can be isolated from both ventral and dorsal E13.5 spinal cord

A critical component of the current understanding of oligodendrocyte development *in vivo* is that specific precursor cells for oligodendrocytes first appear in the ventral spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Timsit et al., 1995; Hall et al., 1996; Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). We subsequently determined whether GRP cells are selectively localized in the ventral spinal cord at or before the time when putative oligodendrocyte precursor cells first appear ventrally. Because previous studies have shown that GRP cells are already present at E13.5 (Rao et al., 1998), we microdissected dorsal and ventral portions of the E13.5 cord to determine the regional distribution of GRP cells; this is a half day earlier than the earliest reported appearance of specific oligodendrocyte precursor cells, as defined by expression of the PDGF receptor (Hall et al., 1996). Freshly isolated cells from dorsal and ventral cord were immunolabeled with A2B5 antibody, purified by fluorescence-activated cell sorting, and plated at clonal density on grid dishes in different conditions as described below. In three independent experiments, the dorsal spinal cord consistently contained an average of 19 ± 8% A2B5$^+$ cells, whereas the ventral portion contained an average of 52 ± 7% A2B5$^+$ cells. Thus, although the ventral cord contained a higher proportion of A2B5$^+$ cells than did the dorsal cord, such cells were found in both regions of the cord.

To determine whether dorsal- and ventral-derived A2B5$^+$ cells

were GRP cells, the A2B5$^+$ clones were first grown in the presence of bFGF until they reached a size of 10–20 cells. Astrocytic differentiation was then induced by exposing cultures for 3 d to 10% FCS. All clones contained both A2B5$^-$GFAP$^+$ type-1 astrocytes and A2B5$^+$GFAP$^+$ type-2 astrocytes independent of their site of isolation. Thus, these cells were typical of GRP cells in their ability to generate two distinct astrocyte populations. Generation of oligodendrocytes was also possible with both ventral- and dorsal-derived cells, as discussed in the following section.

GRP cells derived from both the ventral and dorsal E13.5 spinal cord can generate O2A/OPCs, oligodendrocytes, and astrocytes

Because expression of PDGF receptor-α in the E14 spinal cord has been interpreted to be an indication of a preferential ventral origin of oligodendrocytes (Pringle and Richardson, 1993; Hall et al., 1996), we asked whether ventral- and dorsal-derived GRP cells differed in their ability to generate O2A/OPCs and/or oligodendrocytes. GRP cells were isolated from ventral or dorsal E13.5 spinal cord as described in the preceding section. Freshly isolated cells were plated at a low density on coverslips in the presence of FGF and exposed to conditions (PDGF plus TH) that would induce the transition into O2A/OPCs (as determined previously) or to conditions that would potentially inhibit a transition into O2A/OPCs. As a potential inhibitor molecule, we used bone morphogenetic protein-4 (BMP4), which has been shown to inhibit oligodendrocyte generation (Mabie et al., 1997; Grinspan et al., 2000; Mehler et al., 2000; Zhu et al., 2000) and is present in the embryonic neural tube (D'Alessandro and Wang, 1994; Barth et al., 1999; Grinspan et al., 2000; Liem et al., 2000). After 3 d of *in vitro* growth in the condition discussed, cells were stained with the O4 monoclonal antibody and with anti-GalC and anti-GFAP antibodies.

In the presence of PDGF and TH, cells from both the dorsal and ventral spinal cord were able to generate O4$^+$GalC$^-$ cells with equal frequencies but differed with respect to oligodendrocyte generation (Fig. 2). Specifically, 88 ± 6% of dorsal-derived cells were O4$^+$GalC$^-$, 3 ± 2% were GalC$^+$ oligodendrocytes, and 3 ± 2% were GFAP$^+$ astrocytes. In contrast, 74 ± 9% of ventral-derived cells were O4$^+$GalC$^-$, 28 ± 5% were GalC$^+$ oligodendrocytes, and 2 ± 1% were GFAP$^+$ astrocytes. Thus, although both dorsal and ventral cells were able to generate O2A/OPCs, only ventral-derived cells generated a significant number of GalC$^+$ oligodendrocytes over a 5 d time period. The lack of oligodendrocytes in dorsal cultures is not likely to be attributable to preferential cell death, because the total number of cells was not different in dorsal and ventral cultures (327 ± 8 and 326 ± 38, respectively). In addition, dorsal-derived cells demonstrated an equal ability to eventually generate oligodendrocytes. If cultures were examined after 10 d in the presence of TH, instead of after 5 d, then 69 ± 15% of the ventral cells and 73 ± 3% of dorsal cells were oligodendrocytes (data not shown).

Differences between dorsal- and ventral-derived GRP cells were also observed in response to BMP4. When dorsal- or ventral-derived GRP cells were grown in the presence of BMP4 in concentrations ranging from 1 to 100 ng/ml over 3 d, we observed that BMP4 promoted the generation of astrocytes in both dorsal and ventral cells (Fig. 3). At a low BMP concentration (1 ng/ml), ventral cells were more likely to differentiate into astrocytes than were dorsal cells (45 ± 4% vs 14 ± 4%, respectively). The preferential generation of GFAP$^+$ cells in ventral

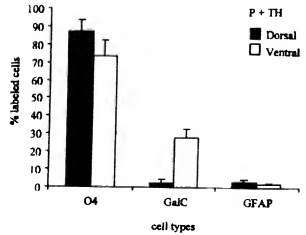

Figure 2. Both dorsal- and ventral-derived GRP cells generate O4$^+$GalC$^-$ cells. A2B5$^+$ cells were isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos and plated in the presence of bFGF supplemented with PDGF plus TH (*P + TH*) for 7 d. Dorsal and ventral cultures were then stained with O4, anti-GalC, and anti-GFAP antibodies. Both dorsal- and ventral-derived cultures generated comparable numbers of O4$^+$ precursor cells. However, GalC$^+$ oligodendrocytes were found predominantly in ventral-derived GRP cell cultures. Only a small fraction of both dorsal and ventral cultures gave rise to GFAP$^+$ astrocytes. Two independent experiments examining six data points for each condition revealed comparable results.

cultures was a transient phenomenon, in that only in dorsal-derived cultures did these numbers increase over the next several days (as discussed in the following paragraph). The addition of 10 ng/ml BMP had an identical effect on ventral and dorsal cells (48 ± 3% and 54 ± 7% astrocytes, respectively). The most dramatic difference between ventral and dorsal cells was observed at high BMP doses (100 ng/ml). In this condition, ventral cells responded with cell death rather than cell differentiation. In contrast, dorsal-derived GRP cells differentiated almost completely into astrocytes when exposed to 100 ng/ml BMP4.

Because BMP4 at 1 ng/ml revealed differences between dorsal- and ventral-derived GRP cells in the absence of toxicity, we subsequently examined the generation of O4$^+$GalC$^-$ cells in this culture condition (Fig. 4*A*). Cells were plated at a low density on coverslips in the presence of FGF and BMP4 (1 ng/ml) and examined after 7 d to allow for the generation of O4$^+$GalC$^-$ cells and/or GalC$^+$ oligodendrocytes. In cultures of GRP cells derived from dorsal spinal cord, the majority of cells (87 ± 8%) differentiated into GFAP$^+$ astrocytes, and only 12 ± 7% of the cells were O4$^+$GalC$^-$. We did not observe any GalC$^+$ oligodendrocytes in these cultures. In contrast, when ventral-derived cells were exposed to 1 ng/ml BMP for 7 d, 47 ± 8% differentiated into astrocytes (as observed for 3 d time point discussed previously) and 52 ± 7% of the cultures consisted of O4$^+$GalC$^-$ cells. Again, we did not observe any GalC$^+$ oligodendrocytes. Thus, BMP4 exposure was associated with a strikingly more significant decrease in the number of O4$^+$GalC$^-$ cells in dorsal- than in ventral-derived GRP cells.

We subsequently determined whether the addition of TH, a potent inducer of the generation of O4$^+$GalC$^-$ cells and/or oligodendrocytes, could counteract the effects of BMP4 (Fig. 4*B*). Dorsal and ventral cells were exposed to BMP at 1 ng/ml in the presence of TH at 50 nM for 7 d before the cultures were labeled

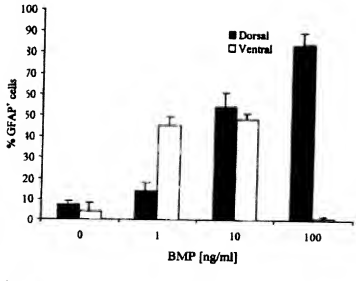

Figure 3. BMP4 induces differentiation of astrocytes from dorsal- and ventral-derived A2B5$^+$ cells in a dose-dependent manner. A2B5$^+$ cells, isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos, were plated at a low density in the presence of bFGF and increasing concentrations of BMP4 (0.1–100 ng/ml). After 3 d, cultures were labeled with anti-GFAP antibodies and the number of astrocytes was determined. Whereas dorsal cultures exhibited a continuous, dose-dependent increase in the number of GFAP$^+$ astrocytes, ventral-derived GRP cells generated significantly more astrocytes at lower doses of BMP (1 ng/ml) at this time point, and higher doses of BMP4 (100 ng/ml) proved to be lethal to ventral-derived GRP cells.

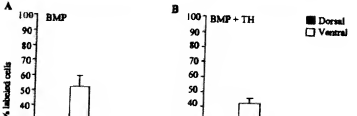

Figure 4. Differential effects of BMP4 on dorsal- and ventral-derived GRP cells. GRP cells were isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos and plated at a low density in the presence of FGF and BMP4 (*A*) (1 ng/ml) or FGF, BMP4 (1 ng/ml), and TH (*B*). To allow for oligodendrocyte generation, cultures were examined after 7 d for the presence of O4$^+$GalC$^-$ precursor cells or GalC$^+$ oligodendrocytes. Although GalC$^+$ oligodendrocytes were only found in ventral GRP cell cultures containing TH, both dorsal- and ventral-derived cultures contained O4$^+$GalC$^-$ precursor cells. In the presence of BMP, the ability of dorsal GRP cells to generate O4$^+$GalC$^-$ precursor cells was lower than that of ventral-derived cultures; this was not changed by the addition of TH.

with O4, anti-GalC, and anti-GFAP antibodies. As shown in Figure 4*B*, the addition of TH had little or no effect on the generation of O4$^+$GalC$^-$ cells in both dorsal and ventral cultures. However, we did detect a small but significant increase ($p < 0.002$) in the number of GalC$^+$ oligodendrocytes specifically in the ventral-derived cells. This effect was not seen in dorsal-derived cultures.

GRP cells can be generated from dorsal and ventral neuroepithelial stem cells of the E10.5 spinal cord

Our results thus far demonstrate that there is a dorsal–ventral gradient in GRP cell distribution in the spinal cord of the E13.5 rat, and that dorsal- and ventral-derived GRP cells are dissimilar in their abilities to generate oligodendrocytes over short time periods *in vitro*. In addition, these two populations differ in their response to BMP. Because GRP cells themselves are derived from neuroepithelial stem cells (NSCs) (Rao and Mayer-Pröschel, 1997), we subsequently determined whether dorsal- and ventral-derived NSCs differed in their capacity to generate GRP cells.

In these experiments, E10.5 spinal cord [at which time point all cells are NSCs (Kalyani et al., 1997)] was microdissected into dorsal and ventral regions. Dissociated cells were plated at clonal density on fibronectin/laminin-coated grid dishes in the presence of 10 ng/ml bFGF and embryonic chick extract (CEE), a condition that prevents differentiation of NSCs (Kalyani et al., 1997). After 3 d in culture, when clones reached a size of 20–50 cells, CEE was removed to allow the clones to differentiate into lineage-restricted precursor cells (Kalyani et al., 1997; Mayer-Pröschel et al., 1997; Rao and Mayer-Pröschel, 1997). After 5 d in the absence of CEE, clones were stained with A2B5 antibody and the number of clones containing A2B5$^+$ cells was determined.

Both dorsal- and ventral-derived NSCs generated A2B5$^+$ cells with a similar efficiency. From a total number of 175 ventral-derived NSC clones, 152 (i.e., 87%) contained A2B5$^+$ cells after 5 d of *in vitro* growth. Similarly, 200 of 213 (84%) dorsal-derived NSC clones cell clones contained A2B5$^+$ cells at this time point. Analysis of the differentiation potential of A2B5$^+$ cells derived from dorsal and ventral NSCs confirmed that these cells expressed the differentiation characteristics of GRP cells (Table 2). These experiments were performed as described previously (Rao and Mayer-Pröschel, 1997). Briefly, clones were stained with A2B5 as live cells and single clones were picked and replated into grid dishes. Single A2B5$^+$ cells were marked and expanded in the presence of bFGF. After clones reached a size of 20–40 cells (5 d), they were switched to 10% FCS to generate astrocytes. After 7 d, clones were stained with A2B5 and anti-

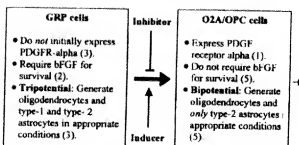

Figure 5. Sequential lineage restriction in the glial lineage of the CNS. A side-by-side comparison of the salient features of two lineage-restricted glial precursors of the CNS is shown. The evidence presented here strongly suggests a progressive and sequential transition from the tripotential GRP cell to the bipotential O2A/OPC. In the developing spinal cord, it currently seems most likely that this transition is controlled in a temporal and spatial pattern and is regulated by cell-extrinsic signaling molecules (Pringle et al., 1992; Rao and Mayer-Pröschel, 1997; Rao et al., 1998).

GFAP antibodies. All astrocyte-containing clones always contained a mixture of type-1 and type-2 astrocytes, regardless of whether they were generated in dorsal- or ventral-derived cultures and whether they were generated in response to FCS (or BMP, data not shown). A smaller number of clones consisted of A2B5$^+$ cells only, and none of these clones contained oligodendrocytes. (Table 2). In contrast, exposure of clones to PDGF plus TH for 10 d was associated with oligodendrocyte generation in all clones. Although differences were not striking, significantly more ventral clones generated oligodendrocytes than did dorsal clones over this time period (35 ± 7% vs 25 ± 2%, respectively; $p < 0.02$).

DISCUSSION

One of the essential challenges that arises with the discovery of any new precursor cell population is to determine how these cells might be integrated into (or might alter) existing views on tissue development. In the present studies on the tripotential GRP cell of the embryonic rat spinal cord, we have found that this recently discovered novel glial precursor cell can generate progeny with the antigenic phenotype and differentiation characteristics of bipotential O2A/OPCs. This process is regulated by cell-exogenous signaling molecules, with growth in the presence of PDGF plus TH being particularly effective in promoting such differentiation. In contrast to previous suggestions that putative oligodendrocyte precursor cells are localized in ventral regions of the E14 spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Timsit et al., 1995; Hall et al., 1996; Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000), GRP cells could be isolated from both the dorsal and ventral cord of E13.5 rats. However, there were differences between dorsal- and ventral-derived GRP cells in their response to conditions that promote or inhibit generation of O2A/OPCs or oligodendrocytes, with ventral-derived GRPs exhibiting a greater propensity to differentiate along the oligodendrocyte lineage.

The demonstration that GRP cells can yield O2A/OPCs integrates these two glial precursor cell populations for the first time and indicates that their relationship is one of sequential lineage restriction rather than being independent precursors that generate oligodendrocytes. In light of our present studies, the simplest model of oligodendrocyte generation that appears to be consistent with all available data would be that production of these cell types requires the initial generation of GRP cells from NSCs followed by the generation of O2A/OPCs from GRP cells (Fig. 5). Our previous studies (Rao and Mayer-Pröschel, 1997; Rao et al., 1998) indicated strongly that GRP cells are a necessary intermediate between NSCs and differentiated glia, and our present studies raise the possibility that O2A/OPCs are a necessary intermediate between GRP cells and oligodendrocytes. Despite the fact that both GRP cells and O2A/OPCs are A2B5$^+$, it seems unlikely that the O4$^+$GalC$^-$ cells studied in our *in vitro* experiments were derived from a subset of A2B5$^+$O4$^-$ bipotential O2A/OPCs present in the original GRP cell culture. In our previous characterizations of GRP cells derived from E13.5 spinal cords, we consistently failed to find clones that gave rise exclusively to type-2 astrocytes when exposed to 10% FCS, even when cells were serially recloned three times over a period of several weeks (Rao et al., 1998). Moreover, analysis of hundreds of putative GRP cell clones thus far has failed to reveal clones that generate only type-2 astrocytes when exposed to FCS or BMPs (Mayer-Pröschel, unpublished observations). Thus, it appears that the generation of cells with the characteristics of O2A/OPCs is a differentiation event that requires exposure of GRP cells to appropriate inductive signals, such as PDGF plus TH. Moreover, we could find no GalC$^+$O4$^-$ oligodendrocytes in any conditions, which would have at least raised the possibility that oligodendrocytes might be generated directly from GRP cells. Such results are consistent with previous observations that passage through an O4$^+$GalC$^-$ stage of development is required for oligodendrocyte generation from bipotential O2A/OPCs (Gard and Pfeiffer, 1990, 1993; Gard et al., 1995). Our data are also consistent with other studies indicating that O4$^+$GalC$^-$ cells are bipotential (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999).

It remains formally possible that GRP cells might be able to generate oligodendrocytes without passage through an intermediate O2A/OPC stage, or that NSCs could generate O2A/OPCs without going through a GRP cell stage. Nonetheless, it is important to stress that no data exist to support the possibility that O2A/OPCs are directly generated from NSCs or that oligodendrocytes are directly generated from either NSCs or GRP cells. Thus, the developmental pathway we suggest is at present the only one supported by experimental observations.

It is of particular interest to find that ventral-derived GRPs seem to differ from dorsal cells in such a manner so as to have an increased probability to generate O2A/OPCs and/or oligodendrocytes, even in the presence of BMP. Thus, it may prove necessary not only to study GRP cells but also to focus attention on ventral-derived GRP cells to understand the mechanism of action of those factors that eventually lead to oligodendrocyte generation. It will be of considerable interest to determine whether these differences are intrinsic to ventral- or dorsal-derived GRP cells or are acquired as a consequence of exposure to particular environmental signals. It also will be of interest to determine whether the O2A/OPCs generated from dorsal and ventral GRP cells themselves differ in their responsiveness to

inducers of oligodendrocyte generation, an interpretation that would be consistent with our data (Fig. 4*B*). In addition, our observation that the responsiveness of GRP cells to PDGF plus TH as promoting signals of O2A/OPC and oligodendrocyte generation may decrease with increased GRP cell expansion *in vitro* is reminiscent of our previous findings that O2A/OPCs expanded for continued periods become less responsive to PDGF as a mitogen (Bogler et al., 1990). Although the biological implications of this observation with respect to GRP cell biology require additional investigation, this result does emphasize the importance of expanding precursor cell populations *in vitro* as minimally as possible in studies on the function of exogenous signaling molecules.

It is important to consider the question of whether all previous studies attempting to define the early origin of the oligodendrocyte lineage have in fact been describing early differentiation events affecting GRP cells. It is clear from our previous work that GRP cells can express the PDGFR without losing their tripotential character (Rao et al., 1998). In addition, our ongoing work (Pröschel, Gass, and Mayer-Pröschel et al., unpublished observations) is demonstrating that GRP cells can also be NG-2$^+$ and GD3$^+$, two other antigens that have been used in studies on O2A/OPCs (Mayer-Pröschel, unpublished observations). Moreover, it currently appears that GRP cells are the dominant (if not exclusive) A2B5$^+$ cell population in the spinal cord until as late as E17 (Mayer-Pröschel, unpublished observations). Thus, it is beginning to seem likely that events such as expression of PDGFR in ventral A2B5$^+$ cells may reflect a differentiation process in GRP cells rather than the transition to being an O2A/OPC. Analyzing the early stages of generation of O2A/OPCs from GRP cells, whether *in vitro* or *in vivo*, will require identification of a marker that can be used to antigenically distinguish GRP cells from the A2B5$^+$O4$^-$ stage of O2A/OPCs. As indicated, none of the markers currently available seem to enable this distinction.

The field of developmental neurobiology is in the early stages of determining the relationship between different lineage-restricted precursor cells in the CNS, and our present experiments represent a critical step in determining whether GRP cells may be the ancestors of all glial populations of the spinal cord. Our present observations are consistent in two ways with such a suggestion. First, if this hypothesis were to be correct, then GRP cells should be able to give rise to O2A/OPCs (as we have found). We also would anticipate that GRP cells would be found in both the dorsal and ventral cord, although they may generate different progeny in these two regions. In future studies, it will be important to discover whether precursor cells with the properties of GRP cells also exist in other regions of the CNS. In addition, it will be important to determine whether other progeny of GRP cells include the A2B5$^+$ astrocyte precursor cells present in embryonic (E17) spinal cord and originally described by Fok-Seang and Miller (1992, 1994), the putative astrocyte precursor cells from the embryonic mouse cerebellum described by Seidman et al. (1997), the astrocyte precursor cells described by Mi and Barres (1999), or the pre-O2A progenitor cell described by Grinspan et al. (1990). In addition, it is of importance to determine whether the developmental inter-relationships that seem to exist in the spinal cord also apply to development of the brain. By identifying the relationship between these developmental pathways and the signals responsible for these transitions, we will move closer to a comprehensive understanding of glial development in the CNS.

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EVIDENCE APPENDIX
EXHIBIT 4

Declaration of Steven A. Goldman under 37 C.F.R. § 1.132

PATENT
Docket No.: 19603/1426 (CRF D-2238A)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: Richard Hutson
Serial No.	:	09/282,239	
Cnfrm. No.	:	To Be Assigned	Art Unit: 1652
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

DECLARATION OF STEVEN A. GOLDMAN UNDER 37 C.F.R. §1.132

U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

I, STEVEN A. GOLDMAN, pursuant to 37 C.F.R. § 1.132, declare:

1. I received B.A. degrees in Biology and Psychology from the University of Pennsylvania in 1978, a Ph.D. degree in Neurobiology from Rockefeller University in 1983, and an M.D. degree from Cornell University Medical College in 1984.

2. I am a Professor of Neurology and Neuroscience at Weill Medical College at Cornell University and an Attending Neurologist at New York Presbyterian Hospital.

3. I am a named inventor of the above patent application.

Kirschenbaum Article

4. I am a co-author of Kirschenbaum, et. al., "*In Vitro* Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Human Forebrain," Cerebral Cortex 6: 576-89 (1994) ("Kirschenbaum").

5. The study described in Kirschenbaum was carried out in my laboratory and I was the senior, supervising scientist on the study; therefore, I fully understand this work. Kirschenbaum cultures samples of adult temporal lobes under conditions suitable for neuronal differentiation, while exposed to ^{3}H-thymidine. These samples were incubated for 7-28 days, stained for neuronal and glial antigens, and autoradiographed. Neuron-like cells were found in explant outgrowths and monolayer dissociates of the subependymal zone and periventricular white matter but not the cortex. A small number of Map-2$^+$ and Map-5$^+$/glial fibrillary acidic protein$^-$ cells did incorporate ^{3}H-thymidine, suggesting neuronal production from precursor mitosis. However, the O4$^+$ oligodendrocytes were postmitotic. Even though the abstract of Kirschenbaum states that "O4$^+$ oligodendrocytes, although the predominant cell type, were *largely* postmitotic (emphasis in original)", I said this only because I am generally reluctant to make conclusions in absolute terms. Nevertheless, it is clear from the following statement on page 582 of Kirschenbaum that, in fact, *all* of the oligodendrocytes were post-mitotic:

> These O4$^+$/GFAP$^\pm$ cells were mitotically quiescent; among a sample of 8044 such cells, culled from four plates of subcortical white matter (2011 ± 858.6 O4$^+$ cells/plate, mean ± SD), *none* incorporated ^{3}H-thymidine *in vitro*, despite the frequent observation of ^{3}H-thymidine-labeled astrocytes in the same plates (emphasis in original).

The failure of the Kirschenbaum study to identify mitotic oligodendrocyte progenitor cells caused me to continue working to identify and produce such cells. These efforts were ultimately successful in producing the invention of the present application.

Bottenstein Patent

6. U.S. Patent No. 5,276,145 to Bottenstein ("Bottenstein") is directed to substantially purified preparations containing a neural progenitor regulatory factor that is important in regulating and coordinating production of oligodendrocytes and type 2 astrocytes. The identification of this factor was carried out with brain cells derived from neonatal rats of 1-3 days of age. These cells represented a mixture of cell types, that included "progenitors", "Type 2 Astrocytes", "Early Oligodendrocytes", "Late Oligodendrocytes", "Total Oligodendrocytes", "Type 1 Astrocytes", and "Microglia".

7. There are fundamental differences between the biology of rat and human oligodendrocyte progenitor cells. These are unaddressed in Bottenstein, which discusses findings

limited to neonatal rat brain. Whereas rat oligodendrocytes appear to retain mitotic potential, human oligodendrocytes do not (see Kirschenbaum). As a result, the oligodendrocyte progenitor cell of the rat brain cannot be considered homologous to its human counterpart. In particular, methods that permit the selective extraction and/or growth of oligodendrocyte progenitors from the rat brain do not differentiate between oligodendrocyte progenitor cells and mature oligodendrocytes able to re-enter the mitotic cycle. In humans, these constitute two discrete phenotypes, lineally related but temporally distinct. Our present invention teaches the selective acquisition of a highly enriched – to virtual purity - mitotically-competent oligodendrocyte progenitor cell pool, operationally separate and distinct from post-mitotic or mature oligodendrocytes.

8. Bottenstein was directed at the enrichment of glial progenitor cells from newborn rat brain. Newborns have an abundant population of still-developing oligodendrocyte progenitor cells that may constitute a significant fraction of all of the cells in neonatal brain tissue. Bottenstein reported that >30% of the cells of its tissue dissociates expressed the marker of this phenotype. With the addition of B104 conditioned media and the neural progenitor regulatory factor, this fraction increased to just over 40%. The nature of these cells is that of a still-mixed pool, in that the following populations appear to be represented by Bottenstein's data: astrocytes, oligodendrocytes, and a mixture of oligodendrodendroglial lineage cells of widely different developmental stages.

9. In contrast to the cells acquired from newborn rats using the Bottenstein protocol, the present invention is achieved with a procedure that permits, in both young and old humans, the selective extraction of progenitor cells strongly biased to oligodendrocytic phenotype, and allows the purification of these cells, including those from tissues in which they are scarce (e.g., postnatal and adult brain tissues harboring <1% of the desired oligodendrocyte progenitor cell type). In Example 5 of the present patent application, we reported the virtual purification of oligodendrocyte progenitor cells from tissues with a P/CNP2 promoter-targeted FACS-defined incidence of <1%. This constituted a far greater enrichment of the oligodendrocyte progenitor cell (i.e. 170-fold) than that achieved by Bottenstein (i.e. less than 1.5-fold) and yields a far more pure product of oligodendrocyte progenitor cells.

10. In contrast to Bottenstein, the human oligodendrocyte progenitor cell populations achieved through our protocols are virtually pure as to phenotype. Compare Figure 5B to its control, Figure 5A. In Figure 5A, the gated single cell represents the false-positive sort incidence. Such incidences constitute <1% of the frequency of events noted in Figure 5B, indicating >99% purity of the P/CNP2:hGFP-sorted oligodendrocyte progenitor cells. This can be modulated as a function of sort speed to achieve any desired degree of purity, the trade-off being lower yields as higher degrees

of purification are achieved. By virtue of the high-purity extraction attainable by fluorescence-activated cell sorting, the progenitor cells we produce are never exposed to paracrine factors released by other cells, after removal from tissue. This permits their maintenance in an undifferentiated and phenotypically-unbiased state, in contrast to the mixed cellular milieu afforded by Bottenstein, in which non-oligodendrocytic and non-glial progenitor-derived phenotypes remain abundant.

11. As a result of these considerations, the selective propagation of mitotically-active oligodendrocyte progenitor cells from the neonatal rat brain, as taught by Bottenstein, does not predict the successful isolation of mitotic oligodendrocyte progenitor cells from postnatal or adult human brain tissue.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 5/31/02

Steven A. Goldman, M.D., Ph.D.

EVIDENCE APPENDIX
EXHIBIT 5

Noble et al., "The O2A (Adult) Progenitor Cell: A Glial Stem Cell of the Adult Central Nervous System," *Seminars in Cell Biol.* 3:413-22 (1992)

Seminars in CELL BIOLOGY, Vol 3, 1992 : pp 413-422

The O-2A^{adult} progenitor cell: a glial stem cell of the adult central nervous system

Mark Noble, Damian Wren† and Guus Wolswijk**

Systematic comparison of the properties of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells derived from optic nerves of perinatal and adult rats has revealed that these two populations differ in many fundamental properties. In particular, O-2A^{perinatal} progenitor cells are rapidly dividing cells capable of generating large numbers of oligodendrocytes over a relatively short time span. Oligodendrocyte differentiation generally occurs synchronously in all members of a clone, thus leading to elimination of that clone from the pool of dividing cells. However, some O-2A^{perinatal} progenitors are also capable of giving rise to O-2A^{adult} progenitors. These latter cells express many of the characteristics of stem cells of adult animals, including the capacity to undergo asymmetric division and differentiation. We suggest that precursors which function during early development give rise to terminally differentiated end-stage cells and to a second generation of precursors with properties more appropriate for later developmental stages. It is this second generation of precursors which express the properties of stem cells in adult animals, and we therefore further suggest that our work offers novel insights into the possible developmental origin of stem cells.

Key words: progenitor / astrocyte / *perinatal* / *adult*

IN THE LIGHT OF the considerable physiological differences between development and maturity, it has seemed likely that precursor cells which contribute to the early generation of a tissue and the precursors involved in replacement of cells in older animals might differ in their properties. The explosive growth of embryogenesis would be inappropriate in most adult tissues, and processes must exist for slowing down this rapid generation of cells. However, precursor populations cannot be entirely eliminated, as there is a need in adult tissues for the maintenance of a population of precursor cells which would have the capacity to contribute to tissue repair. It is these cells which include the stem cells of adult tissue.

For at least several different cellular lineages, substantial differences have indeed been demonstrated between the precursor cells present during development and in the adult animal. For example, there are fundamental differences between embryonic myoblasts and adult muscle satellite cells,[1,2] and between fetal and adult cells of both the haematopoietic and sympathoadrenal lineages.[3,4] In addition, although optic nerves of both perinatal and adult rats contain progenitor cells which can be induced to differentiate *in vitro* into either oligodendrocytes or type-2 astrocytes,[5-10] the oligodendrocyte-type-2 astrocyte (O-2A) progenitors isolated from optic nerves of adult rats differ from their perinatal counterparts in antigenic phenotype, morphology, cell cycle time, motility and time-course of differentiation *in vitro*.[7-10]

In this review we will discuss our attempts to understand the comparative biology of the precursor cells of developing and mature organisms. These studies have led us to propose functional distinctions between precursors which provide the basis for tissue formation during early development (e.g. neuroepithelial stem cells, embryonic stem cells), but which are not maintained in the animal throughout life, and those which are able to provide a source of new cells in tissues of mature animals. At least for the lineage we have examined, our studies have provided several novel insights into the origin and functional biology of stem cells of adult animals.

The O-2A^{perinatal} progenitor

The first step in our studies on glial development in the CNS was the discovery that cultures derived from white matter tracts of the CNS contained two distinct astrocyte populations, termed type-1 and type-2 astrocytes.[11] These two cell types could be readily distinguished from each other on the basis of morphology, antigenic phenotype and response to growth factors. Most importantly, we found that

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optic nerves of perinatal rats contained a population of glial precursors which did not express glial fibrillary acidic protein (GFAP) at the time of isolation, but which could be induced to become GFAP+ type-2 astrocytes by growth in tissue culture.

Subsequent studies on the precursors of type-2 astrocytes led to the discovery that these cells could also be induced to differentiate into oligodendrocytes.[5] Oligodendrocytic differentiation of O-2A progenitors occured when progenitors were grown in chemically-defined medium and did not require the presence of inducing factors. In contrast, astrocytic differentiation required the presence of appropriate inducing factors, at least one of which is found in fetal sera of a number of different species.

Initial studies on oligodendrocytic differentiation of O-2A progenitors isolated from optic nerves of perinatal rats presented the paradox that the cells we were studying were isolated at a time of maximal division of this lineage *in vivo*,[12,13] yet cells did not divide in tissue culture. Resolution of this paradox began with the discovery that cortical astrocytes promoted O-2A^{perinatal} progenitor division *in vitro*.[14] The astrocytes used in these studies express many of the properties of type-1 astrocytes of the optic nerve, which are the first identifiable glial cells to appear in the nerve.[15] The similarity of these two populations led us to suggest that type-1 astrocytes were responsible for supplying the mitogen(s) required to keep O-2A progenitors in division *in vivo*. Moreover, populations of O-2A^{perinatal} progenitors grown in the presence of purified cortical astrocytes were capable of undergoing extended division while also continuing to generate more oligodendrocytes,[14] a pattern of behaviour similar to that occurring *in vivo*. Thus, the failure of O-2A^{perinatal} progenitors to divide in our initial *in vitro* studies was due to the lack of necessary mitogens, which appeared to be supplied by another glial cell type of the optic nerve.

Further studies demonstrated that purified cortical astrocytes could also promote the correctly timed differentiation *in vitro* of O-2A^{perinatal} progenitors isolated from optic nerves of embryonic rats.[16] The molecular mechanism by which this timing is controlled remains a mystery, although all evidence to date indicates that it is the O-2A^{perinatal} progenitors themselves which are measuring elapsed time.[17,18] A potential linkage between the measurement of elapsed time by dividing cells and the control of differentiation has also been observed for fibroblasts and haematopoietic stem cells (for review see ref 19). In the case of O-2A^{perinatal} progenitors, it appears that this biological clock causes clonally related dividing progenitors to differentiate synchronously into oligodendrocytes within a limited number of cell divisions.[10,17,18] However, it is not yet known whether the mechanism which underlies this synchronous differentiation of clones of dividing cells is also responsible for the first appearance of oligodendrocytes in the rat optic nerve at the day of birth *in vivo*, or the equivalent time *in vitro*.

The effects of purified cortical astrocytes, and of type-1 astrocytes from the optic nerve, on O-2A^{perinatal} progenitor division *in vitro* appear to be mediated by platelet-derived growth factor (PDGF).[18,20-22] O-2A^{perinatal} progenitors exposed to either PDGF or astrocyte-conditioned medium exhibited a bipolar morphology, migrated extensively (with average migration rates of $24.6 \pm 5.6\ \mu m\ h^{-1}$) and divided with an average cell cycle length of 20 ± 6 h. PDGF was also as potent as type-1 astrocytes at promoting the correctly timed differentiation *in vitro* of embryonic O-2A progenitors into oligodendrocytes.[18] Moreover, antibodies to PDGF blocked the mitogenic effect of type-1 astrocytes on embryonic O-2A progenitor cells, causing these cells to cease division and to differentiate prematurely even when growing on monolayers of type-1 astrocytes. Thus, this single mitogen was able to elicit a complex behavioural phenotype from O-2A^{perinatal} progenitors which included normal functioning of the cellular mechanisms involved in the measurement of elapsed time. Interestingly, recent studies have indicated that neurons, which also promote division of O-2A^{perinatal} progenitors *in vitro*,[22,23] may also be a source of PDGF.[24,25] However, the specific contributions of either neuronal or astrocytic[26] production of PDGF to the development of the O-2A lineage *in vivo* is not yet known.

O-A^{adult} progenitors

To attempt to gain insights into the cellular mechanisms underlying regeneration of the oligodendrocyte population following demyelinating damage *in vivo*, we also initiated studies on O-2A progenitors of the adult CNS. In our initial studies, which again were focused on the rat optic nerve, we found that O-2A progenitors isolated from adult animals differed from their perinatal counterparts in several ways. When co-cultured with purified cortical astrocytes, O-2A^{adult} progenitors had a unipolar morphology *in vitro*,[7] whereas O-2A^{perinatal} progenitors were usually bipolar.[7,27] In

addition, O-2A^{adult} progenitors had a longer average cell cycle time *in vitro* than O-2A^{perinatal} progenitors (65 ± 18 h versus 18 ± 4 h),[7,20] migrated more slowly ($4.3 \pm 0.7\ \mu m\ h^{-1}$ versus $21.4 \pm 1.6\ \mu m\ h^{-1}$),[7,27] and take longer to differentiate (3-5 days versus 2 days for 50% differentiation).[7] Furthermore, O-2A^{adult} progenitors stimulated to divide by purified cortical astrocytes were O4$^+$ while dividing O-2A^{perinatal} progenitors were O4$^-$ (ref 7; I. Sommer, M. Noble, unpublished observations).

The appearance of adult-specific precursors in any lineage raises questions about their developmental origin. Are these cells derived from a common ancestor cell which, for example, initially gives rise to O-2A^{perinatal} progenitors, and then gives rise to O-2A^{adult} progenitors during later stages of development? Alternatively, are perinatal and adult precursor populations derived from two distinct ancestors, despite being specialized to produce similar terminally differentiated end-stage cells?

The continued presence of precursor populations in adult animals also raises questions about how such populations are maintained within any particular tissue throughout life. The maintenance of a precursor population throughout life is generally thought to be associated with the presence of a stem cell population which supplies new cells to the precursor pool for use in cell replacement following normal turnover or injury. For example, it has been suggested that the presence of proliferating O-2A progenitors in the adult animal requires the existence of a pre-progenitor, or stem cell, compartment in the O-2A lineage.[6] The requirement for a stem cell compartment to support the prolonged maintenance of dividing O-2A progenitors in the nerve is further indicated by the self-extinguishing nature of the O-2A^{perinatal} progenitor population. As described earlier, O-2A^{perinatal} progenitors grown *in vitro* in the presence of purified cortical astrocytes (as a source of PDGF)[14,20] generally divide and differentiate symmetrically, such that all members of a clonal family of cells synchronously differentiate into oligodendrocytes within a limited number of divisions.[17,18] It is clear that this mode of division and differentiation is incompatible with continued self-renewal of precursors throughout life, and it was thus not surprising to find that O-2A^{perinatal} progenitors are present only in small numbers in cultures prepared from optic nerves of 1-month-old rats[8] and are not detectable in cultures prepared from optic nerves of adult rats.[7]

Generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors

Analysis of the development of O-2A^{adult} progenitor cells in cultures derived from 3-week-old rats, the age when the relative proportion of perinatal to adult O-2A progenitors appears to be changing most rapidly *in vivo*,[8] has indicated that some O-2A^{perinatal} progenitor-like cells have the ability to generate O-2A^{adult} progenitor-like cells when co-cultured with purified cortical astrocytes.[10] These experiments were carried out by filming the behaviour of families of cells derived from single O-2A progenitors. Due to the simplicity of the optic nerve cultures, and our extensive characterization of the cell types found in these cultures, the morphological information provided in these films could be used to identify with great precision O-2A progenitors, and to distinguish between cells with *perinatal*- or *adult*-like phenotypes.

In seven individual time-lapse microcinematographic films, with a total analysis of 15 separate families of O-2A lineage cells, we found four examples of families in which (1) the founder cell gave rise at the first division to two cells with the characteristic morphology, cell-cycle length and motility of O-2A^{perinatal} progenitors and (2) subsequent to the first division, members of the family expressed the unipolar morphology, lengthened division times and slow migration rates typical of O-2A^{adult} progenitors.

Figure 1 depicts diagramatically the history of one of the families wherein O-2A^{perinatal} progenitor-like cells were seen to give rise to O-2A^{adult} progenitor-like cells. In this family the founder cell first generated two further O-2A^{perinatal} progenitor-like cells (cells a and b). The family branch represented by one of these progenitors (cell a) terminated, over two divisions, with the production of three oligodendrocytes (cells c, d and e, which were characterized by their multipolar morphology, lack of division and lack of migratory behaviour).[20,31] The other branch (cell b) first produced three further perinatal progenitor-like cells before all of these cells started to express longer cell cycle times and migration rates. By the next division, all of the motile and dividing members of this family expressed a unipolar morphology, a cell cycle length of > 40 h ($x = 45$ h) and a migration rate of $\leqslant 6\ \mu m\ h^{-1}$ ($x = 4\ \mu m\ h^{-1}$); see cells f, g and h in Figure 1. Similar observations were made in the other three families in which a *perinatal*-to-*adult*-transition was observed.[10]

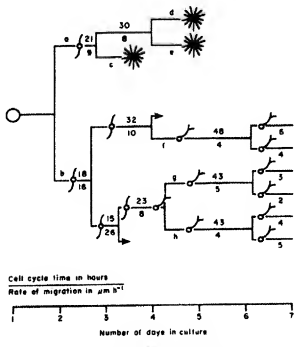

Figure 1. Bipolar O-2A*perinatal* progenitor-like cells that divide and migrate at a fast rate give rise to unipolar O-2A*adult* progenitor-like cells which migrate and divide more slowly. Fifteen clonal colonies of O-2A progenitors, stimulated to divide by cortical astrocyte-derived mitogens, were followed by time-lapse microcinematography. Within 15 colonies suitable for detailed analysis, four clear examples were found of O-2A*perinatal* progenitor-like cells that were bipolar, were highly motile and had a short cell cycle time, which in the first division generated more O-2A*perinatal* progenitor-like cells, and which eventually gave rise to cells which expressed the unipolar morphology, lengthened cell cycle time and slow migration rate of O-2A*adult* progenitors. One of the families in which dividing O-2A*perinatal* progenitor gave rise to O-2A*adult* progenitor-like cells is represented diagramatically in the figure. The morphology of a progeny cell is indicated in the figure only when the cell was clearly bipolar, unipolar, or oligodendrocyte-like. Since some progeny cells moved out of the field of photography (depicted with an arrow) their fate could not be determined. The numbers above the lines are the cell cycle times in hours, while the numbers below the lines are the migration rates in $\mu m\ h^{-1}$. The transitions shown could not be ascribed to changes in the composition of the tissue culture medium, since all still cultures contained actively dividing and migrating O-2A*perinatal* progenitor-like cells at the end of the filming period.

Our time-lapse observations suggest that the transition from *perinatal* to *adult* phenotype is not an abrupt one, in that generation of O-2A*adult* progenitor-like cells may require two or more cell divisions, with the cells present after one division expressing cell-cycle times and motility characteristics intermediate between the *perinatal* and *adult* phenotypes. These results also are consistent with our previous studies on the characteristics of cells derived from optic nerves of 1-week to 1-month-old rats, in which we observed cells with phenotypes which could not be classified unambiguously as *adult*-like or *perinatal*-like.[8]

Extended self-renewal in the O-2A lineage is associated with the *in vitro* generation of O-2A*adult* progenitors

To test further the hypothesis that O-2A*adult* progenitors might be derived from O-2A*perinatal* progenitors, we then serially passaged perinatal optic nerve cells over the course of 3 months. In these experiments, optic nerve populations containing O-2A*perinatal* progenitors, but not O-2A*adult* progenitors, were passaged onto fresh monolayers of purified and irradiated cortical astrocytes for up to six passages.

Serial passaging of O-2A progenitors derived from optic nerves of perinatal rats was associated with a shift in the progenitor population from entirely *perinatal*-like to predominantly *adult*-like, as judged by antigenic and morphological criteria and by changes in the population doubling times.[10] In the early passage cultures, dividing O-2A progenitor-like cells (identified by [^{3}H]-thymidine labelling, immunolabelling and autoradiography) expressed the bipolar morphology and A2B5$^+$O4$^-$ antigenic phenotype characteristic of O-2A*perinatal* progenitor cells. In contrast, >80% of the dividing O-2A progenitors in the later passage cultures expressed the O4$^+$ antigenic phenotype characteristic of O-2A*adult* progenitors, and 92% of these cells also expressed the characteristic unipolar morphology of O-2A*adult* progenitors.[7] The rate of increase in the numbers of new progenitors and oligodendrocytes in these cultures also decreased significantly with increasing passage number, and fell from the 24 h doubling times characteristic of perinatal populations to approach the long doubling times characteristic of adult populations. In agreement with the increase in the average doubling time with increasing passage number, the proportion of progenitor-like cells which incorporated [^{3}H]-thymidine during a 24 h pulse decreased with successive passages.

The results of our serial passaging experiments were thus consistent with the hypothesis that O-2A*adult* progenitors are derived from O-2A*perinatal*

progenitors and further suggested that expression of the capacity for prolonged self-renewal in this lineage is associated with the appearance of O-2A^{adult} progenitors. The mechanism which might underly such self-renewal was suggested by observations that serial passaging was associated with a slight increase in the proportion of colonies which contained both oligodendrocytes and [^{3}H]-thymidine labelled O-2A progenitors, an observation examined in closer detail using cells derived from adult animals (see next section).

Characteristics of division and differentiation in colonies of O-2A^{adult} progenitor cells

As O-2A^{perinatal} progenitors are not detected at all in cultures derived from adult optic nerves, it is unlikely that generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors is the mechanism which allows maintenance of the *adult* progenitor in the nerve throughout life. The slight increase in the proportion of O-2A lineage colonies containing both oligodendrocytes and [^{3}H]-thymidine labelled O-2A progenitors, seen in our passaging experiments, raised the possibility that O-2A^{adult} progenitors might be able to divide and differentiate asymmetrically. Such a pattern of division and differentiation would allow these cells to give rise to more progenitors and generate oligodendrocytes at a slow rate. To examine this possibility under conditions which would allow cells to undergo several divisions, we analysed the composition and size of oligodendrocyte-containing colonies generated from O-2A^{perinatal} and O-2A^{adult} progenitors grown at clonal densities (< 1 cell/30 mm^2) on monolayers of purified cortical astrocytes (to promote progenitor division).[7,14] As the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors (as would occur in cultures derived from 3-week-old rats) would have complicated analysis of these experiments, the optic nerve cells used in these experiments were obtained exclusively from newborn and adult rats. Colonies were examined after a length of time which would allow cells to undergo $\leqslant 6$ divisions and $\leqslant 10$ divisions, this being 6 and 10 days for O-2A^{perinatal} progenitors and 15 and 25 days for O-2A^{adult} progenitors, respectively.

Oligodendrocyte-containing colonies

As in previous experiments,[17] the composition and size of oligodendrocyte-containing colonies derived from O-2A^{perinatal} progenitors were consistent with the view that the generation of oligodendrocytes by these cells is associated with symmetric division and clonal differentiation. Sixty-six percent of the oligodendrocyte-containing colonies examined on Day 10 consisted entirely of oligodendrocytes and, even as early as Day 6, the A2B5$^+$GalC$^-$ progenitor-like cells in mixed colonies were most frequently multipolar non-dividing cells (i.e. not labelled with [^{3}H]-thymidine) which appeared to have been visualized just prior to oligodendrocytic differentiation. Only 7% of the oligodendrocyte-containing colonies derived from *perinatal* progenitors and visualized on Day 6, and 14% of those visualized on Day 10, contained both oligodendrocytes and dividing progenitor cells (as judged by the incorporation of [^{3}H]-thymidine). Moreover, oligodendrocyte-containing colonies derived from O-2A^{perinatal} progenitors clustered around sizes of 2, 4, 8, 16, 32, 64 and 128 cells/colony at 6, 8 and 10 days after plating, as expected when clonally-related cells divide symmetrically and differentiate synchronously.

Unlike the results obtained with O-2A^{perinatal} progenitors, the composition of oligodendrocyte-containing colonies derived from individual dividing O-2A^{adult} progenitors was consistent with the hypothesis that the generation of oligodendrocytes by these cells occurred by means of asymmetric division and differentiation.[10] Over 75% of the oligodendrocyte-containing colonies derived from individual O-2A^{adult} progenitors grown at clonal densities contained both oligodendrocytes (which generally do not divide in these tissue culture conditions)[7,14] and [^{3}H]-thymidine-labelled progenitors after both 15 and 25 days of *in vitro* growth, periods of time which would allow $\leqslant 6$ or $\leqslant 10$ average cell cycles for O-2A^{adult} progenitors.[7] The proportion of colonies which contained both oligodendrocytes and radiolabelled O-2A progenitor cells on Days 15 and 25 of *in vitro* growth was very similar, even though the average size of the oligodendrocyte-containing colonies continued to increase with time in culture (from a median value of 7 cells/colony on Day 15 to a median value of 11 cells/colony on Day 25). Only 10% of the colonies visualized on Day 25 consisted entirely of oligodendrocytes, and the remaining 14% contained oligodendrocytes and progenitors which were unlabelled by [^{3}H]-thymidine. In addition sizes of oligodendrocyte-containing colonies did not cluster at factors of 2 on either Day 15 or Day 25 of *in vitro* growth.

Oligodendrocyte-free colonies

Sixty-two percent (110/176) of the colonies derived from O-2A^{adult} progenitors contained no oligodendrocytes even after 25 days of *in vitro* growth. Oligodendrocyte-free colonies seen at this stage were generally small, and over 80% of these colonies (89/110) contained $\leqslant 16$ cells at Day 25. Consistent with the small size of many of these colonies, $<20\%$ (23/110) of the oligodendrocyte-free colonies contained any cells wich were labelled by a 20 h pulse with [^{3}H]-thymidine. In contrast, in colonies derived from O-2A^{perinatal} progenitors, only 30% (41/136) of the colonies were free of oligodendrocytes on Day 10 *in vitro*.

The O-2A^{adult} progenitor as a stem cell

While the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors provides a possible explanation for the origin of the *adult* cell, the lack of O-2A^{perinatal} progenitors in adult optic nerve[7] suggests that other mechanisms are involved in maintenance of a dividing population of O-2A^{adult} progenitor in the adult animal. Although it has been previously suggested[6] that the presence of such cells in the adult requires the existence of an ancestral stem cell, capable of generating O-2A lineage cells throughout life, several observations now raise the possibility that the O-2A^{adult} progenitors may themselves function as stem cells.

The first stem-cell like property of O-2A^{adult} progenitors derives from the observation that this population is maintained in the rat optic nerve as a dividing population seemingly throughout life (ref 6; G. Wolswijk, E. Abney, unpublished observations). O-2A^{adult} progenitor-like cells can be isolated from optic nerves during the first week after birth and such cells remain in the nerve for at least the first year of life, in contrast with O-2A^{perinatal} progenitors, which have largely disappeared from the optic nerve by one month after birth.[7,8] *In vitro* observations suggest that O-2A^{perinatal} progenitors would disappear from the nerve as a consequence of symmetric differentiation of most clones of cells into oligodendrocytes and differentiation of the remaining cells into O-2A^{adult} progenitors (and possibly type-2 astrocytes, although the *in vivo* existence of these cells is controversial; see refs 28-32).

The second stem-cell like property of O-2A^{adult} progenitors is their long (60-65 h) cell cycle times.[7-9] Our most recent studies [10] further suggest that the population of O-2A^{adult} progenitors may even contain a sizeable proportion of cells with cell cycle times in excess of 100 h. Examination of colonies developing *in vitro* over 25 days showed that the great majority (89/110) of these colonies contained $\leqslant 16$ cells after 25 days *in vitro*, and that only a small proportion (23/110) of these colonies contained cells which were labelled with a 20 h pulse of [^{3}H]-thymidine. Both of these results are consistent with the existence of O-2A^{adult} progenitors with very long cell-cycle times.

Also of potential relevance to the question of whether O-2A^{adult} progenitors express stem-cell like characteristics are our observations consistent with the view that these cells can undergo asymmetric division and differentiation *in vitro*. Unlike colonies derived from O-2A^{perinatal} progenitors, oligodendrocyte-containing colonies derived from O-2A^{adult} progenitors generally contained O-2A^{adult} progenitors which were labelled by [^{3}H]-thymidine, indicating that onset of differentiation in the *adult* progenitor-derived colonies was not associated with cessation of cell division in the whole colony. The capacity to undergo asymmetric division and differentiation is an important attribute of *bona fide* stem cells of adult animals.

A further stem-cell like feature displayed by *adult* progenitors grown *in vitro* was that a far higher proportion of oligodendrocyte-containing colonies than oligodendrocyte-free colonies contained O-2A^{adult} progenitors labelled with a 20 h pulse of [^{3}H]-thymidine (75 versus 20%; ref 10). Similarly, the onset of differentiation of epidermal stem cells into keratinocytes in any clone of cells is associated with an increased likelihood of finding cells engaged in DNA synthesis, in association with passage of stem cell progeny through a transit amplifying population of cells engaged in differentiation.[33]

Growth factor co-operation and self-renewal in the O-2A lineage

All of the research described thus far was carried out in cultures in which O-2A progenitor division was promoted either by purified cortical astrocytes or by PDGF (the progenitor mitogen secreted by these cells). However, we have also found that there are other developmental programmes which can be expressed by dividing O-2A progenitors. As will be discussed below, some of these findings may be of particular relevance to understanding the control of

precursor self-renewal and also to the elicitation from adult stem cells of a pattern of growth likely to be of importance in responding to tissue injury.

O-2A^{perinatal} progenitors division can be induced by exposure to cells to basic fibroblast growth factor (bFGF), but cells induced to divide by this mitogen were multipolar and showed little migratory behaviour.[34] In addition, cells induced to divide by bFGF had a cell-cycle length of 45 ± 12 h, in contrast with the 18 ± 4 h cell cycle length elicited by exposure to PDGF. These results indicate that PDGF and bFGF function in the O-2A lineage as modulators of differentiation as well as functioning as promoters of cell division. PDGF and bFGF also differ in their effects on oligodendrocytes themselves, in that only bFGF is able to promote division of these cells.[34-36]

The effect of bFGF on oligodendrocytic differentiation of O-2A^{perinatal} progenitors is currently a subject of controversy. In our initial studies, we found that O-2A progenitors exposed to bFGF differentiated prematurely to form oligodendrocytes.[34] In contrast, other investigators found that this same mitogen inhibited differentiation of purified O-2A^{perinatal} progenitors.[37] The several methodological differences between the two sets of studies (ranging from the source of progenitors to the methods of tissue culture) make it difficult to determine the reasons for these differing observations. Our more recent studies do however suggest that at least part of the discrepancy between the two sets of results may have been due to effects of other factors present in the cultures, and that bFGF does indeed inhibit oligodendrocytic differentiation of purified O-2A^{perinatal} progenitors.[46]

In respect to O-2A ^{perinatal} progenitors, the most intriguing results of our studies with PDGF and bFGF was the discovery that progenitors exposed simultaneously to these two mitogens continued to divide without differentiating into oligodendrocytes.[34] For example, cultures prepared from optic nerves of 19-day-old rat embryos began to generate oligodendrocytes after 2 days when established in the presence of PDGF alone,[18,34] yet remained oligodendrocyte-free even after 10 days of growth in the presence of PDGF + bFGF.[34] Further experimentation has demonstrated that O-2A^{perinatal} progenitors can be grown continually for a year or more *in vitro* as long as cells are continually exposed to both of these mitogens (S.C. Barnett, M. Noble, unpublished observations). O-2A^{perinatal} progenitors grown in this manner retain the ability to undergo oligodendrocytic differentiation when removed from the presence of both mitogens. We do not yet know whether O-2A^{perinatal} progenitors grown for extended periods in this manner will generate O-2A^{adult} progenitors.

The discovery that cooperation between growth factors can cause prolonged self-renewal of precursors revealed a previously unkown means of regulating self-renewal in a precursor population. Such cooperation may, however, represent a more general phenomenon, as indicated by the importance of growth factor cooperation in promoting the extended division *in vitro* of haematopoietic stem cells[38] and primordial germ cells.[47] It will be of considerable interest to determine the extent to which cooperation between different growth factors is responsible for eliciting particular aspects of stem cell behaviour.

Growth factor cooperativity and lesion repair

While it is difficult to determine the role (if any) played by PDGF/bFGF cooperativity during development, some of our most recent studies have suggested that such cooperativity may be of profound importance in the context of lesion repair. These studies have also revealed a further property of O-2A^{adult} progenitors of relevance in considering the stem cell-like behaviour of these cells.

We have recently found that simultaneous exposure of O-2A^{adult} progenitors to PDGF + bFGF converts many of these cells to a rapidly dividing and highly motile phenotype with a bipolar morphology and antigenic phenotype very similar to that expressed by O-2A^{perinatal} progenitors.[48] Thus, these cells can be induced to express a phenotype which seems likely to be of relevance to repair of demyelating lesions. These findings demonstrate that the molecular mechanisms which underlie the characteristic behaviour of O-2A^{perinatal} progenitors are not irreversibly inactivated with the generation of O-2A^{adult} progenitors, but are instead placed under the control of slightly different signalling processes than those which function in the perinatal cells. The finding that rapid cell division can be induced in O-2A^{adult} progenitors is consistent with observations that repair of virally-induced demyelination *in vivo* appears to be preceded by increases in the numbers of O-2A^{adult} progenitor-like cells.[39] In addition, studies in other laboratories have suggested an increased production of FGFs and PDGF following CNS damage.[40-42] It is particularly intriguing, however, that our studies also suggest that the ability of O-2A^{adult} progenitors to maintain a

rapidly dividing and migrating phenotype is not maintained beyond a small number of divisions, suggesting intrinsic limitations may exist in the extent to which these cells are capable of contributing to myelin repair.[48] Such a possibility is reminiscent of claims that MS lesions are initially repaired, but eventually become permanently demyelinated.

A revised view of the O-2A lineage

Figure 2 summarizes some of our current views about development of the O-2A lineage, in which the population of O-2A^{perinatal} progenitors is now seen as tripotential and capable of giving rise to oligodendrocytes, type-2 astrocytes and O-2A^{adult} progenitors. Our studies suggest that O-2A^{perinatal} progenitors express the properties of true progenitor cells, in that these cells generally express a limited life-span before undergoing differentiation (at least when stimulated by purified cortical astrocytes or PDGF). However, a previously unanticipated differentiation pathway which appears to be open to O-2A^{perinatal} progenitors is to give rise to a new generation of precursors, these being the stem cell-like O-2A^{adult} progenitors. The apparent development of O-2A^{adult} progenitors, with stem cell-like characteristics, from a rapidly dividing perinatal population differs significantly from the pattern of development seen in other lineages, where slowly dividing stem cells (of developed, rather than developing, tissues) have been seen to give rise to rapidly dividing progenitors (for review see ref 33). However, no other studies have focused on the origin of potential stem populations in the manner in which we have.

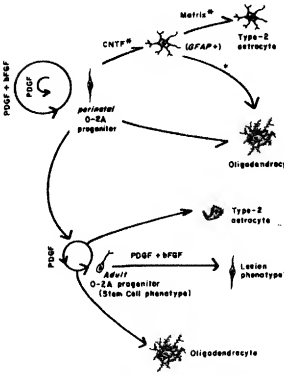

Figure 2. The O-2A lineage, as presently perceived in our laboratory. *Ciliary neurotrophic factor (CNTF) is thought to initiate astrocytic differentiation of O-2A progenitors, as detected by expression of glial fibrillary acidic protein (GFAP). Full differentiation of O-2A^{perinatal} progenitors into type-2 astrocytes, however, requires the additional presence of an unidentified extracellular matrix component (see refs 43, 44). Differentiation of O-2A^{adult} progenitors into type-2 astrocytes has not yet been extensively studied, although we have observed that the type-2 astrocytes generated following growth in serum-containing medium do not express the stellate morphology of type-2 astrocytes derived from O-2A^{perinatal} progenitors.[7] For more detailed discussion on other aspects of differentiation in this lineage see also refs 21, 29, 45.

A general hypothesis on the origin of stem cells, which we believe emerges from our studies, is as follows: precursors which function during early development express properties required for cells participating in the initial creation of a tissue. Such properties are inappropriate at later developmental stages, at least for some tissues. In such instances, the early precursors give rise to a second generation of precursors with properties more appropriate to later development, as well as to terminally differentiated end-stage cells. It is this second generation of precursors which represent the stem cells of adult animals. Unfortunately, it will first be necessary to be able to distinguish unambiguously between fetal (or perinatal) and adult precursor cells in other tissues before it can be determined whether phenomena similar to those observed in the O-2A lineage also occur in other lineages.

In regards to the O-2A lineage itself, there are many challenging questions which remain unanswered. It will first be important to determine whether the process we have described for the O-2A lineage of the optic nerve occurs in all regions of the CNS. On a more fundamental level, it will be necessary to determine the relationship between the symmetric and asymmetric O-2A^{perinatal} progenitors. Are these two cell types distinct from the beginning of their existence, is one cell type the ancestor of the other, or do they represent two possible differentiation pathways of a still earlier ancestor cell? Moreover,

although it seems likely that it is the asymmetrically behaving O-2A^{perinatal} progenitors which eventually give rise to O-2A^{adult} progenitors, the mechanism which causes the earlier cells to generate cells of an *adult* phenotype is a mystery. At present, we know that O-2A^{perinatal} and O-2A^{adult} progenitors exposed to platelet-derived growth factor (PDGF) each express their characteristic morphologies, migratory properties and cell cycle lengths.[9,20] It will be an important challenge to define the molecular alterations which allow a single cell-signalling molecule to elicit such different behaviours from these two precursor populations, and to determine whether these alterations are alone sufficient to convert O-2A^{perinatal} progenitors into O-2A^{adult} progenitors. It is also interesting that this replacement of an O-2A^{perinatal} progenitor population by an O-2A^{adult} progenitor population *in vitro* is at least superficially similar to that which occurs *in vivo* (albeit over a slightly shorter time-scale than that seen *in vitro*). The ability to reproduce such a conversion in tissue culture will facilitate future studies on molecular mechanisms which might be involved in the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors. Finally, the O-2A^{adult} progenitor cell may offer a suitable model system for probing the molecular mechanisms involved in the generation of asymmetric division and differentiation.

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EVIDENCE APPENDIX
EXHIBIT 6

Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cell Isolates Myelinate the Congenitally Dysmyelinated Brain," *Nature Medicine* 10:93-97 (2004)

nature medicine

Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain

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Both late-gestation and adult human forebrain both contain large numbers of oligodendrocyte progenitor cells (OPCs). These cells may be identified by their A2B5$^+$PSA-NCAM$^-$ phenotype (positive for the early oligodendrocyte marker A2B5 and negative for the polysialylated neural cell adhesion molecule). We used dual-color fluorescence-activated cell sorting (FACS) to extract OPCs from 21- to 23-week-old fetal human forebrain, and A2B5 selection to extract these cells from adult white matter. When xenografted to the forebrains of newborn shiverer mice, fetal OPCs dispersed throughout the white matter and developed into oligodendrocytes and astrocytes. By 12 weeks, the host brains showed extensive myelin production, compaction and axonal myelination. Isolates of OPCs derived from adult human white matter also myelinated shiverer mouse brain, but much more rapidly than their fetal counterparts, achieving widespread and dense myelin basic protein (MBP) expression by 4 weeks after grafting. Adult OPCs generated oligodendrocytes more efficiently than fetal OPCs, and ensheathed more host axons per donor cell than fetal cells. Both fetal and adult OPCS phenotypes mediated the extensive and robust myelination of congenitally dysmyelinated host brain, although their differences suggested their use for different disease targets.

A broad range of pediatric leukodystrophies and storage diseases manifest with myelin failure or loss. Recent studies have focused on the use of transplanted oligodendrocytes or their progenitors to treat congenital myelin diseases. The myelinogenic potential of implanted brain cells was first noted in the shiverer mouse[1,2]. Shiverer is an autosomal recessive mutation; *shi/shi* homozygotes fail to develop MBP or compact myelin and die by 20–22 weeks. Transplanted fetal brain cells[3–6], primary[7] and immortalized[8] neural progenitors, and enriched glial progenitor cells[9] can all myelinate shiverer axons, albeit typically with low efficiency. Similarly, rodent subventricular zone progenitors can engraft another dysmyelinated mutant, the myelin-deficient rat, after perinatal administration[10,11]. Indeed, all of these studies suggest the feasibility of myelinating congenitally dysmyelinated brain, even though none of the cell sources used did so efficiently.

On this basis, we asked whether highly enriched populations of OPCs directly isolated from the human brain might be used as more effective substrates for cell-based therapy of congenital dysmyelination. Specifically, we postulated that human OPCs, whether derived from the fetal brain during its period of maximum oligoneogenesis, or from the adult subcortical white matter[12,13], could mediate large-scale myelination of a congenitally dysmyelinated host. We report here that both fetal and adult human OPCs, highly enriched by surface antigen-based FACS, were capable of widespread and high-efficiency myelination of the shiverer mouse brain after perinatal xenograft. We also report significant differences in the behavior of fetal and adult-derived OPCs, which suggests that they may be useful in treating different specific disease targets.

Cells dissociated from the late second-trimester human ventricular zone (21–23 weeks gestation) were first magnetically sorted to isolate A2B5$^+$ cells[13–16], including oligodendrocytic and neuronal progenitor cells. Because PSA-NCAM is expressed by immature neurons at this stage of development[17], we then used FACS to deplete PSA-NCAM$^+$ neurons from the larger A2B5$^+$ cell population. This yielded a subpopulation of A2B5$^+$PSA-NCAM$^-$ cells, which defined our oligodendrocyte progenitor pool. Two-color FACS showed that the A2B5$^+$PSA-NCAM$^-$ fraction constituted 15.4 ± 4.8% of the cells in samples from the 21- to 23-week ventricular zone ($n = 5$; **Supplementary Figure 1** online). Of these A2B5$^+$PSA-NCAM$^-$ cells, 76.1 ± 0.5% expressed oligodendrocytic O4 by 1 week after FACS, whereas only 7.5 ± 0.3% expressed astrocytic glial fibrillary acidic protein (GFAP) and only 2.0 ± 1.3% expressed neuronal β-III tubulin. These data support the glial restriction and oligodendrocytic bias of sorted A2B5$^+$PSA-NCAM$^-$ cells. Because we achieved higher net yields with immunomagnetic separation of A2B5$^+$ cells followed by FACS depletion of NCAM$^+$ cells, compared with two-color FACS, we used this technique for progenitor isolation.

Homozygous *shi/shi* mice were injected intracallosally with fetal progenitor cell isolates on either their day of birth (P0) or on postnatal day 1 (P1), and later killed at 4, 8, 12 or 16 weeks of age. None of the animals were immunosuppressed; we relied on perinatal tolerization to ensure graft acceptance[18,19]. The injections resulted in substantial engraftment, defined as ≥100 cells per coronal section at three rostrocaudal levels sampled >100 mm apart, in 34 of the 44 neonatal mice injected for this study (25 of 33 injected with fetal human OPCs, and 9 of the 11 injected with adult-derived OPCs). By 12 weeks of age, the recipients showed donor engraftment throughout the callosum and capsular and commissural

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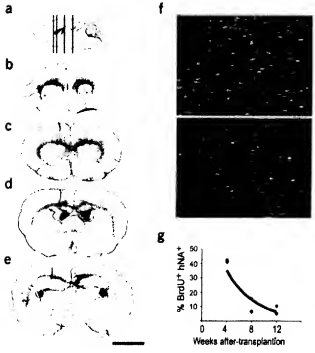

Figure 1 Fetal human OPCs disperse rapidly to infiltrate the forebrain. (**a–e**) Human cells were localized by immunostaining for hNA. Low-power fluorescence images of coronal section of forebrain (**b–e**) were collected at representative anteroposterior levels as indicated in schematic (**a**, ref. 25). Engrafted cells are shown in red (**b–e**). (**f**) Immunofluorescent detection of BrdU (green) and hNA (red) 4 (top) and 12 (bottom) weeks after xenograft of human OPCs into shiverer mice. Arrows indicate mitotic human OPCs ($BrdU^+hNA^+$). (**g**) Regression plot of mitotically active donor cells as a function of time after perinatal implant. Rate of BrdU incorporation declined according to the exponential regression $y = 83.4e^{-0.22x}$, with correlation coefficient $r = -0.87$ ($P = 0.012$). Scale bar, 3 mm (**b–e**) or 50 μm (**f**).

white matter, extending caudally to the basis pontis (**Fig. 1a–e**). During this time, cell division among the engrafted progenitors, though initially high at 4 weeks, fell to relatively low and stable levels by 8 and 12 weeks (Fig. 1f,g). The fraction of human donor cells that incorporated BrdU during the 48 h before mice were killed dropped from 42 ± 6.1% at 4 weeks to 8.2 ± 2.4% at 12 weeks.

During this same period, many of the fetal progenitors matured into myelinogenic oligodendrocytes, as indicated by their expression of MBP. At 4 weeks, no MBP was detectable in 10 of 11 animals, despite widespread cell dispersion; scattered MBP^+ cells were noted in one mouse. At 8 weeks, patchy foci of MBP expression were noted in four of seven mice, and by 12 weeks, widespread MBP expression was noted throughout the forebrain white matter tracts in five of seven mice. By this time, the engrafted mice typically expressed MBP throughout the entire corpus callosum, as well as throughout the fimbria and internal capsules (**Fig. 2a–d**). Because shiverer mice express only the first exon of the *Mbp* gene[2], and hence have no immunodetectable MBP, any MBP detected in these recipients was necessarily donor-derived[8]. In addition, optical sectioning confirmed that the MBP^+ cells were of human origin, in that each MBP^+ profile was associated with a human nuclear antigen $(hNA)^+$ soma (**Fig. 2c,e–h**).

We next asked whether donor-derived myelin effectively wrapped host axons. We used confocal imaging and electron microscopy to assess axonal ensheathment and myelin compaction, respectively. Confocal analysis was first done on the brains of three shiverer mice that were implanted on P1 with 100,000 fetal human OPCs each, and sacrificed at 12 weeks. Foci of dense MBP expression were assessed by confocal imaging, after immunolabeling for hNA and neurofilament (NF) protein to detect donor-derived cells and host shiverer axons, respectively. We found

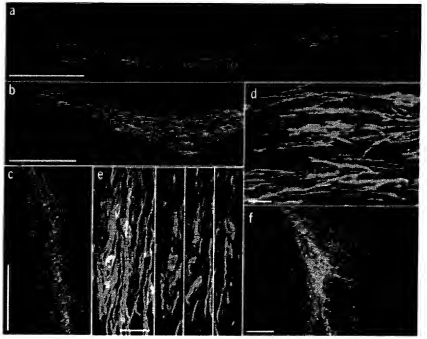

Figure 2 Engrafted human OPCs myelinate an extensive region of the forebrain. (**a**,**b**) MBP expression (green) by sorted human fetal OPCs implanted into homozygous shiverer mice. Large regions of the corpus callosum were myelinated by 12 weeks. **a** and **b** are two different mice. (**c**) Human OPCs migrated to and myelinated fibers throughout the dorsoventral extents of the internal capsules, resulting in widespread forebrain remyelination after a single perinatal injection. (**d**) MBP expression (green) in engrafted shiverer mouse callosum 3 months after perinatal xenograft was associated with human hNA^+ donor cells (red). (**e**) Confocal optical sections of implanted shiverer mouse callosum, with hNA^+ donor cells (red) surrounded by MBP (green). Human cells (arrows) were found within meshwork of MBP^+ fibers. Right three images taken 1 μm apart, were merged to form left image. (**f**) Striatocallosal border of shiverer mouse brain, 3 months after perinatal engraftment with human fetal OPCs (blue). Donor-derived MBP^+ oligodendrocytes and myelin (red) are evident in the corpus callosum, while donor-derived $GFAP^+$ astrocytes (green) predominate on the striatal side. Scale bar, 1 mm (**a–c**), 100 μm (**d**), 20 μm (**e**) or 200 μm (**f**).

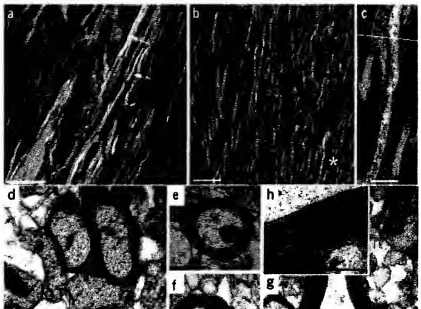

Figure 3 Axonal ensheathment and myelin compaction by engrafted human OPCs. (**a**) Confocal micrograph showing triple immunostain for MBP (red), human nuclear antigen (HNA; blue) and NF (green). All MBP immunostaining is derived from sorted human OPCs, whereas NF$^+$ axons are those of mouse host. Arrows indicate segments of mouse axons ensheathed by human oligodendrocytic MBP. (**b**) Composite of optical sections through corpus callosum of shiverer recipient killed 12 weeks after fetal OPC implantation. (**c**) Higher magnification of area indicated by * in **b**. MBP immunoreactivity (red) surrounds ensheathed axons (green) on both sides. (**d**) Electron micrographs of sagittal section through corpus callosum of adult *shi/shi* homozygote. Shiverer axons typically have single loose wrapping of uncompacted myelin, such that major dense lines do not form. (**e**–**h**) Representative electron micrographs of 16-week-old homozygous shiverer mice implanted with human OPCs shortly after birth. These images show resident shiverer axons with densely compacted myelin sheaths. **h**, enlargement of area indicated by * in **g**. Major dense lines are visible between myelin lamellae, providing electron microscopic confirmation of myelination by engrafted human OPCs. Scale bar, 20 μm (**a**,**b**), 5 μm (**c**) or 1 μm (**d**–**h**); **d**, **f**, **g** use bar in **e**.

that the human progenitors generated myelinating oligodendrocytes in great numbers. Of the recipients scored, 11.9 ± 1.6% (mean ± s.e.m.) of NF$^+$ host callosal axons were surrounded by MBP immunoreactivity (n = 3 mice; three fields scored per animal; Fig. 3a–c). We next used electron microscopy to verify that host axons were fully ensheathed by donor-derived oligodendrocytes, and that the latter generated compact myelin. Because MBP is required to compact consecutive layers of myelin together, its expression is required for the major dense line of mature myelin. Myelin in MBP-deficient shiverer mice did not show more than a few loose wrappings and lacked major dense lines (Fig. 3d), whereas *shi*/*shi* graft recipients showed compact myelin with major dense lines (Fig. 3e–h). In a sample of MBP$^+$ fields (n = 50) derived from two mice killed 16 weeks after perinatal implant, 7.4% of callosal axons (136 of 1,832 sampled) had donor-derived myelin sheaths, as defined ultrastructurally by their major dense lines. Thus, engrafted fetal human OPCs efficiently differentiated into myelinogenic oligodendrocytes.

Some transplanted fetal OPCs differentiated into GFAP$^+$ astrocytes as early as 4 weeks after implantation. In white-matter regions sampled on the basis of high donor-cell engraftment, 12.7 ± 4.3% of fetal donor-derived cells expressed astrocytic GFAP at 12 weeks, and 10.2 ± 4.4% of donor cells expressed MBP. No heterotopic β-III tubulin- or MAP-2-defined neurons of donor derivation were noted at 4, 8 or 12 weeks after implant (n = 33 total). Nevertheless, 40.3 ± 4.2% of donor cells expressed S100-β, which is expressed by astrocytes and young oligodendrocytes, and nestin was expressed by 47.3 ± 4.2%, suggesting that a large proportion of donor cells persisted as glial progenitors after engraftment. Fetal OPCs were recruited as oligodendrocytes or astrocytes in a context-dependent manner, giving rise to both oligodendrocytes and fibrous astrocytes in the presumptive white matter, but only to GFAP$^+$ astrocytes in the gray matter (Fig. 2f and Supplementary Fig. 2 online).

We next asked whether adult-derived OPCs differed from their fetal counterparts with respect to their dispersal, myelinogenic capacity, or time courses thereof. We implanted two litters of P0 shiverer mice with A2B5-sorted OPCs extracted from adult human subcortical white matter. The mice were killed after 4, 8 or 12 weeks, and their brains were stained for hNA and either MBP or GFAP. Nine of 11 mice were successfully engrafted. The adult OPCs achieved widespread and dense MBP expression by 4 weeks (Fig. 4a–d); at 12 weeks, 39.5 ± 16.3% of adult OPCs expressed MBP. In contrast, none of the hNA$^+$ fetal donor OPCs expressed MBP 4 weeks after engraftment, and only 10.2 ± 4.4% did so by 12 weeks ($P < 0.001$ by two-tailed t-test comparing the proportion of MBP$^+$ cells in fetal and adult-derived grafts; Fig. 4a–c). These results indicate that engrafted adult OPCs were at least four times more likely to become oligodendrocytes and develop myelin than their fetal counterparts. Essentially no adult OPCs became astrocytes in the recipient white matter (none developed GFAP expression), whereas 12.7 ± 4.3% of fetal OPCs did so by 12 weeks. Thus, whereas nominally oligodendrocytic progenitors derived from the fetal brain acted as glial progenitors, adult OPCs behaved in a more restricted manner, largely generating either myelinogenic oligodendrocytes or persistent progenitors in recipient white matter. The more rapid myelination by adult OPCs was reflected ultrastructurally, as the major dense lines of compact myelin were readily evident in mice 6 weeks after implantation with adult OPCs at birth (Fig. 4e). No such evidence of myelin compaction was noted in mice implanted with fetal OPCs until 12–16 weeks postnatally.

Despite the apparent competitive advantage of adult OPCs, substantially more fetal than adult donor cells became engrafted in the recipient brains (Fig. 4f). At the midline of the corpus callosum, the region of maximal engraftment, we scored 1,123 ± 205.6 hNA$^+$ fetal donor cells/mm^2. Of these, 117 ± 43.7 were MBP$^+$, and 9.8 ± 3.1% of fetal donor cells dif-

ferentiated into myelinating oligodendrocytes by 12 weeks. In contrast, only 244 ± 182.1 donor cells/mm^2 were noted in the callosal midline of shiverer mice implanted with adult OPCs. Yet 81 ± 59.7, or 38.9 ± 12.9%, of these cells had developed into MBP$^+$ oligodendrocytes by 12 weeks ($P < 0.001$ by two-tailed t-test comparing the proportion of MBP$^+$ cells in fetal and adult grafts; **Fig. 4g**). In addition, whereas 12.7 ± 4.3% of fetal donor cells matured to express GFAP, no adult donor cells gave rise to GFAP$^+$ astrocytes, again suggesting a stronger bias toward the oligodendrocytic phenotype by the adult progenitors. Thus, besides maturing more quickly than fetal OPCs, adult OPCs gave rise to oligodendrocytes in much higher proportions than their fetal counterparts.

To assess whether adult and fetal OPCs differ in the extent to which they ensheath axons, we scored the numbers of axons myelinated by each donor OPC, as defined by confocal-verified MBP$^+$ wrapping of NF$^+$ axons. These absolute values were then expressed as ratios to total number of donor cells and to donor-derived MBP$^+$ oligodendrocytes per field. When assessed 12 weeks after perinatal graft, adult-derived OPCs ensheathed many more host axons per donor cell than their fetal counterparts, an effect that persisted even after we limited our analysis to the number of ensheathed axons per MBP$^+$ donor cell (**Fig. 4h**). In each case, the difference between fetal and adult donor ensheathment efficiency was significant by Mann-Whitney analysis ($P < 0.02$). Thus, adult-derived OPCs matured to ensheath more axons per donor cell than their fetal counterparts.

These results indicate that isolates of human OPCs sorted from the highly oligoneogenic, late second-trimester forebrain, as well as from adult subcortical white matter, can broadly myelinate the shiverer mouse brain, a genetic model of perinatal leukodystrophy. When introduced as highly enriched isolates, both fetal and adult-derived OPCs spread widely throughout the presumptive white matter, ensheathed resident mouse axons and formed antigenically and ultrastructurally compact myelin. Donor-derived myelinogenesis was geographically extensive and was observed throughout all white matter regions of the telencephalon. After implantation, the mitotic expansion of the cells slowed over time (**Fig. 1g**), and neither undesired phenotypes nor parenchymal aggregates were generated. Both fetal and adult-derived OPCs were capable of remyelinating mouse axons, and neither generated heterotopic neurons. We also noted some marked differences between fetal and adult-derived OPCs. Whereas fetal OPCs were highly migratory, they myelinated slowly and inefficiently, and cogenerated astrocytes in recipient white matter as readily as they did myelinogenic oligodendrocytes. In contrast, adult OPCs migrated over shorter distances, but myelinated more rapidly and in higher proportions than did their fetal counterparts, with virtually no astrocytic coproduction. On an individual basis, each adult OPC–derived oligodendrocyte ensheathed and myelinated substantially more axons than did its fetal-derived counterparts (**Fig. 4g**).

Together, these observations suggest that isolates of human glial progenitor cells may provide effective cellular substrates for remyelinating the congenitally dysmyelinated or hypomyelinated brain. In practical terms, the choice of stage-defined cell type may be dictated by both the availability of donor material and the specific biology of the disease target. Their differences notwithstanding, fetal and adult-derived human OPC isolates were capable of achieving widespread and efficient myelination of the dysmyelinated brain, suggesting new strategies for the treatment of the congenital leukodystrophies and myelin disorders.

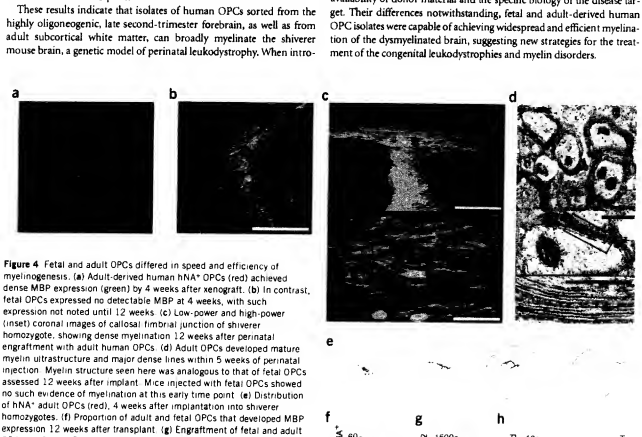

Figure 4 Fetal and adult OPCs differed in speed and efficiency of myelinogenesis. (**a**) Adult-derived human hNA$^+$ OPCs (red) achieved dense MBP expression (green) by 4 weeks after xenograft. (**b**) In contrast, fetal OPCs expressed no detectable MBP at 4 weeks, with such expression not noted until 12 weeks. (**c**) Low-power and high-power (inset) coronal images of callosal fimbrial junction of shiverer homozygote, showing dense myelination 12 weeks after perinatal engraftment with adult human OPCs. (**d**) Adult OPCs developed mature myelin ultrastructure and major dense lines within 5 weeks of perinatal injection. Myelin structure seen here was analogous to that of fetal OPCs assessed 12 weeks after implant. Mice injected with fetal OPCs showed no such evidence of myelination at this early time point. (**e**) Distribution of hNA$^+$ adult OPCs (red), 4 weeks after implantation into shiverer homozygotes. (**f**) Proportion of adult and fetal OPCs that developed MBP expression 12 weeks after transplant. (**g**) Engraftment of fetal and adult OPCs. *, $P < 0.05$; **, $P < 0.005$ by two-tailed Student t-test. (**h**) Numbers of MBP ensheathed NF$^+$ axons per fetal or adult donor OPC, measured as a function both of total donor cell number (left) and MBP$^+$ donor-derived oligodendrocytes (right). *, $P < 0.02$ for fetal compared with adult OPCs, by Mann-Whitney analysis. Scale bar, 100 μm (**a**,**b**), 1 mm (**c**); 30 μm (inset) or 1 μm (**d**).

METHODS

Cells. Fetal OPCs were extracted from 21- to 23-week-old human fetuses obtained at abortion. The forebrain ventricular and subventricular zones were dissected free and chilled on ice. The minced samples were dissociated using papain and DNase as described[20,21]. After washing with 3 h of extraction, and maintained overnight in DMEM/F12/N1 with 20 ng/ml fibroblast growth factor. Adult-derived OPCs were collected from subcortical white matter samples obtained at surgery, as described[12,13]. The eight adult tissue samples used were derived largely from patients undergoing temporal lobe resection for medication-refractory epilepsy. No tissues were accepted from patients with known neoplastic disease. Both fetal and adult samples were obtained with consent, using protocols approved by the institutional review boards of Cornell–New York Presbyterian Hospital, and the Albert Einstein College of Medicine and Jacobi Hospital.

Sorting. The day after dissociation, cells from fetal samples were incubated in a 1:1 ratio with monoclonal antibody A2B5 supernatant (clone 105, American Type Culture Collection) for 30 min, then washed and labeled with fluorophore- or microbead-tagged rat antibody to mouse IgM (Miltenyi Biotec). In some instances, two-channel FACS was used to define the proportions and homogeneity of A2B5- and PSA-NCAM-defined subpopulations, using a FACSVantage SE/Turbo (Becton Dickinson) as described[13,21]. For preparative sorting before transplantation, A2B5$^+$ cells were prepared by magnetic separation (Miltenyi Biotec) according to the manufacturer's protocol. The bound cells were eluted and incubated with mouse antibody to PSA-NCAM (1:25; PharMingen) for 30 min, then with phycoerythrin-tagged secondary antibody (1:200). The PSA-NCAM$^+$ population was then removed by FACS, leaving a highly enriched pool of A2B5$^+$PSA-NCAM$^-$ cells. This PSA-NCAM immunodepletion step was omitted for adult samples, which were sorted on the basis of A2B5 only[12,20]. After sorting, both fetal and adult cells were maintained for 1–7 d in DMEM/F12/N1 with 20 ng/ml basic fibroblast growth factor (20 ng/ml) until implantation.

Transplantation and tagging. Homozygous shiverer mice were bred in our colony. Within 1 d of birth, pups were cryoanesthetized for cell delivery. Donor cells (1×10^5) in 2 μl of HBSS were injected through a pulled glass pipette and inserted through the skull into the presumptive corpus callosum. Transplants were directed to the corpus callosum at a depth of 1.0–1.2 mm, depending on the weight of the pup, which varied from 1.0 to 1.5 g. Pups were killed 4, 8, 12 or 16 weeks thereafter. For some experiments, recipient mice were injected for with bromodeoxyuridine (BrdU; 100 μg/g as a 1.5 mg/100 μl solution) every 12 hours 2 d before killing.

Immunohistochemistry. Transplanted cells were identified using antibody 1281 to human nuclei (Chemicon), monoclonal antibody 91 to cyclic nucleotide phosphodiesterase (CNP) protein (Sternberger and Meyer), rabbit antibody to S-100 (Sigma), rabbit antibody to human nestin (gift of H. Okano, Keio University), Sternberger monoclonal antibody 311 to NF, Sternberger monoclonal antibody 21 to human GFAP, rat antibody to BrdU (Harlan) and either Sternberger monoclonal antibody 94 to MBP or rat antibody 7349 to MBP (Abcam), all as described[7,7,20–22].

Confocal and electron microscopy. Confocal imaging was done using an Olympus Fluoview mated to an IX70 inverted microscope, as described[22]. Argon laser lines were used to achieve three-channel immunofluorescence detection of fluorescein-, Texas red– and Cy5-tagged antibodies; the latter was then pseudocolored blue for presentation. For confocal quantification of ensheathment efficacy, shiverer axons were scored as ensheathed when yellow under lines intersected NF$^+$ axon abutted on each side by MBP immunoreactivity. The proportion of ensheathed axons was defined as the incidence of MBP$^+$/NF$^+$ axons divided by the total number of NF$^+$ axons in each field. For electron microscopy, animals were perfused and post fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in 6% sucrose, then Vibratome-sectioned as alternating thick (400 μm) and thin (100 μm) sections. The latter were immunostained for MBP. Thick sections adjacent to thin sections with MBP expression were then processed in 1% osmium and 1.5% ferricyanide, stained with 1.5% uranyl acetate, embedded in Epon, cut as 100-nm thin sections onto formvar-coated grids, stained with lead citrate and visualized using a JEOL 100 electron microscope[21].

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the Nature Medicine website for details).

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EVIDENCE APPENDIX
EXHIBIT 7

Third Declaration of Steven A. Goldman under 37 C.F.R. § 1.132

PATENT
Docket No.: 19603/4230 (CRF D-2238A)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: Richard Hutson
Serial No.	:	09/282,239	
Cnfrm. No.	:	To Be Assigned	Art Unit: 1652
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

THIRD DECLARATION OF STEVEN A. GOLDMAN UNDER 37 C.F.R. §1.132

Mail Stop
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, STEVEN A. GOLDMAN, pursuant to 37 C.F.R. § 1.132, declare:

1. I received B.A. degrees in Biology and Psychology from the University of Pennsylvania in 1978, a Ph.D. degree in Neurobiology from Rockefeller University in 1983, and an M.D. degree from Cornell University Medical College in 1984.

2. I am a Professor and Chief of the Division of Cell and Gene Therapy of the Department of Neurology, University of Rochester Medical Center, Rochester, New York, where I am the Glenn-Zutes Chair in Biology of the Aging Brain.

3. I am a named inventor of the above patent application.

4. I am familiar with U.S. Patent No. 5,726,145 to Bottenstein ("Bottenstein") and U.S. Patent No. 6,361,996 to Rao, et. al., ("Rao").

5. Rao et al. (Rao et al., "Glial-Restricted Precursors are Derived from Multipotential Neuroepithelial Stem Cells," *Dev. Biol.* 188:48-63 (1997), attached hereto as

Exhibit 1) clearly demonstrate the strong astrocytic bias of their cells, which generated few, if any, oligodendrocytes.

6. There are fundamental differences between the lineage restriction and potential of neonatal and adult oligodendrocyte progenitor cells (Noble et al., "The O2A (Adult) Progenitor Cell: A Glial Stem Cell of the Adult Central Nervous System," *Seminars in Cell Biol.* 3:413-22 (1992), attached hereto as Exhibit 2; and Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cells Effectively Myelinate Dysmyelinated Brain," *Nature Medicine* (January, 2004) (in press), attached hereto as Exhibit 3, which has been accepted for publication (see attached Exhibit 4)). These biological differences between perinatal and adult progenitor cells were not recognized by Rao or Bottenstein, whose cells were restricted to neonatal rodent derivation.

7. Rat oligodendrocyte progenitors are neither biologically nor phenotypically homologous to human oligodendrocyte progenitor cells. Specifically, rat oligodendrocyte progenitors and oligodendrocytes both express the antigenic marker recognized by monoclonal antibody O4. In contrast, this marker is expressed by human oligodendrocytes and their immature forms, but NOT by mitotic oligodendrocyte progenitor cells (See Armstrong et al., "Pre-Oligodendrocytes from Adult Human CNS," J. Neurosci. 12: 1538-47, 1992; Gogate et al., "Plasticity in the Adult Human Oligodendrocyte Lineage," *J. Neurosci.* 14:4571-87 (1994), attached hereto as Exhibit 5; Kirschenbaum et al., "*In vitro* Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Human Forebrain," *Cerebral Cortex* 6: 576-89 (1994); Roy et al., "Identification, Isolation, and Promoter-Defined Separation of Mitotic Oligodendrocyte Progenitor Cells From the Adult Human Subcortical White Matter," *J. Neurosci.* 19: 9986-95 (1999) ("Roy, 1999"), attached hereto as Exhibit 6). As a result, human oligodendrocyte progenitor cells cannot be acquired through the use of O4 as a selection marker, and O4-defined human oligodendroglial cells cannot act as mitotically-competent progenitor cells. This is in sharp distinction to the rat brain, in which the use of this marker can identify oligodendrocyte progenitors. Neither Rao nor Bottenstein recognized the non-applicability of this marker to the separation of human oligodendrocyte progenitor cells. In humans, mitotic cells biased strongly towards the oligodendrocyte lineage are instead recognized by the antigenic phenotype $O4^{-}$/PSA-$NCAM^{-}$/$A2B5^{+}$, which comprise a distinct subpopulation in which the CNP2 promoter is transcriptionally activated (Roy, 1999; Windrem et al., "Progenitor Cells

Derived from the Adult Human Subcortical White Matter Disperse and Differentiate as Oligodendrocytes Within Demyelinated Regions of the Rat Brain," *J. Neurosci. Res.* 69:966-75 (2002), attached hereto as Exhibit 7; Nunes et al., "Identification and Isolation of Multipotential Neural Progenitor Cells from the Subcortical White Matter of the Adult Human Brain," *Nature Med.* 9: 439-47 (2003); attached hereto as Exhibit 8).

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 12-17-03

Steven A. Goldman, M.D., Ph.D.

EVIDENCE APPENDIX
EXHIBIT 8

Kirschenbaum et al., "*In Vitro* Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Forebrain," *Cerebral Cortex* 6:576-89 (1994)

In vitro Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Human Forebrain

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Departments of [1] Neurology and Neuroscience and [2] Neurosurgery, Cornell University Medical College, New York, New York 10021

It has traditionally been held that the adult brain is incapable of significant self-repair, due in part to its inability to generate new neurons. Nevertheless, rodents and birds have been found to harbor neural precursor cells in adulthood. We asked whether the adult human brain might retain such precursors, by culturing samples of temporal lobe under conditions permissive for neuronal differentiation, while exposed to ^{3}H-thymidine. Adult human temporal lobe cultures, derived from cortex, subcortex, and periventricular subependymal zone (SZ), were incubated for 7–28 d, stained for neuronal and glial antigens, and autoradiographed. Neuron-like cells were found in explant outgrowths and monolayer dissociates of SZ and periventricular white matter, but not cortex; they expressed neuronal antigens including MAP-2, MAP-5, NF, and N-CAM, and were GFAP$^-$. Neurons responded to K$^+$ depolarization with rapid and reversible increases in intracellular Ca^{2+}, with much greater increments than those noted in glia. Although most neurons were not ^{3}H-thymidine labeled, a small number of MAP-2$^+$ and MAP-5$^+$/GFAP$^-$ cells did incorporate ^{3}H-thymidine, suggesting neuronal production from precursor mitosis. Rare ^{3}H-thymidine$^+$ neurons were also found in cultures of subventricular white matter; in these, GFAP$^+$ astrocytic mitogenesis was common, while O4$^+$ oligodendrocytes, although the predominant cell type, were largely postmitotic. Thus, the adult human forebrain harbors precursor cells that retain the potential for neuronal production and differentiation *in vitro*.

Among adult mammals, forebrain neurogenesis is highly restricted, both spatially and phylogenetically (Altman and Das, 1966; Korr, 1980; Sturrock, 1982), and has not previously been found in primates (Rakic, 1985; Eckenhoff and Rakic, 1988). In contrast, neurogenesis is widespread and robust in the adult songbird telencephalon, which continues to generate neurons from mitotic ependymal or subependymal (SZ) precursor cells (Goldman and Nottebohm, 1983). [In the adult songbird, it remains unclear whether the precursor cell resides in the ventricular ependyma or one cell below, in the subependyma. As a result, our use of the abbreviation SZ in this article, for all species discussed, encompasses both the nominally defined ependymal and subependymal layers (Boulder Committee, 1970)]. Like the songbirds, lower vertebrates including both teleost fish (Anderson and Waxman, 1985) and lizards (Lopez-Garcia et al., 1988) have been shown to exhibit persistent neurogenesis in adulthood. We previously established a preparation by which neurogenesis could be studied in long-term explant cultures of the adult songbird forebrain (Goldman, 1990). In these explants, the number of neurons generated *in vitro* varied as an inverse function of the serum level, indicating that serum might harbor or induce factors that are antimitogenic for SZ precursor cells (Goldman et al., 1992b). On this basis, we postulated that the lack of neuronal production by non-neurogenic adult brain might result not from an absence of appropriate precursors, but rather from their tonic inhibition by either serum-borne or hormonally stimulated, locally derived agents.

Recent reports have demonstrated the presence of such neuronal precursor cells in cultures derived from adult brain. Reynolds and Weiss (1992) reported epidermal growth factor (EGF)–stimulated neurogenesis in cultures of the adult mouse striatum, while Richards et al. (1992) also described neuronal production in cultures of the adult mouse forebrain, under the influence of basic fibroblast growth factor (bFGF). Ronnett et al. (1990) observed the proliferation of neural cells derived from megalencephalic human brain; however, the transformation state and functional capability of these cells are unclear. Although the source of the neuronal precursors was not established in these reports, the characteristic pattern of ventricular zone neurogenesis in mam-

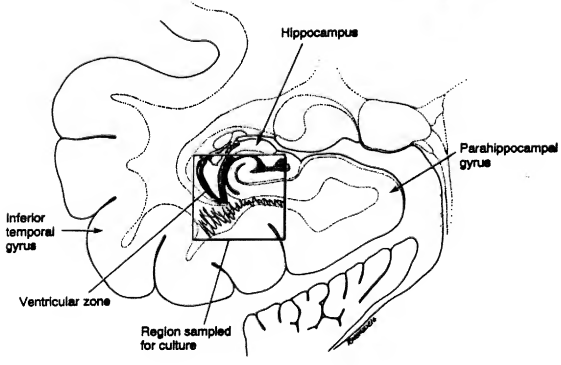

Figure 1. Adult human temporal lobe was obtained from refractory epileptics during anterior temporal lobectomy. This drawing shows a coronal section at a level roughly corresponding to the average posterior border of these resections. The borders of a typical inferior temporal lobe sample (typically from the nondominant hemisphere) are outlined as drawn. This includes the anterior aspect of the parahippocampal gyrus, tempora-occipital portion of the inferior temporal gyrus, and in some cases the hippocampus itself, all underlying the inferior aspect of the temporal horn of the lateral ventricle. From each resection, tissue pieces were dissected into cortical, subcortical, and SZ samples, the latter including both the ependymal surface and adjacent subependymal zone.

malian embryogeny, as well as in the adult avian brain, suggested that neuronal precursors may reside in the adult mammalian forebrain SZ. Indeed, SZ cells continue to divide in the adult mouse brain, but their progeny generally die within 24 hr after mitosis (Morshead and van der Kooy, 1992), although some degree of neuronal differentiation and survival may occur, particularly among cells destined for the olfactory bulb (Altman and Das, 1966; Kaplan and Hinds, 1977; Corotto et al., 1993; Luskin, 1993). In contrast, once removed into explant culture, the adult murine SZ demonstrates both the migration and differentiation of newly generated neurons (Lois and Alvarez-Buylla, 1993). These results suggested the persistence in adults of an SZ progenitor cell population, which remains actively neurogenic in selected groups and brain regions, but which more generally becomes vestigial, yielding short-lived or rare progeny. On this basis, we postulated that the adult human forebrain might harbor such vestigial precursor cells, which retain the capacity for neurogenesis when raised *in vitro*. To test this proposition, we sought evidence of neurogenesis in cultures of adult human temporal lobe. We report here that cells derived from the SZ and periventricular white matter of the adult human forebrain can indeed generate and differentiate into neurons in culture.

Aspects of this work have been reported previously in abstract form (Goldman et al., 1993; Kirschenbaum et al., 1993).

Materials and Methods

Tissue Samples

Adult human temporal lobe was obtained during anterior temporal lobectomy, done for the treatment of medically refractory epilepsy (n = 11 patients, 15–52 years old: four males, and seven females). No tissues were obtained from tumor of any origin, because of the potential danger in confusing proliferating neuroepithelial cells with neoplastic cells *in vitro*. No tissues were used that would otherwise have not been taken as a requirement of surgery. Tissue pieces were dissected into cortical, subcortical, and periventricular samples, the latter including the ependyma and subependymal zone (again, jointly denoted as SZ). The SZ was demarcated by ligature at resection, and dissected from subjacent white matter to a depth of approximately 300 μm (Fig. 1).

Culture Preparation

Each tissue sample was cut into roughly 0.3 mm^3 pieces, which were either cultured directly as explants on laminin, or dissociated for single-cell monolayer culture. Dissociate cultures were prepared by incubating pieces for 40 min in 0.25% trypsin, 1 mM EDTA at 37°C, with intermittent trituration. After pelleting and resuspension in media, cells were plated at roughly 2 × 10^5 cells/ml into either 35 mm petri dishes (0.7 ml/plate) or 24-well plates (0.4 ml/well), which had been coated with human fibronectin (GIBCO–Bethesda Re-

search Labs; 1 mg/cm²). Representative pieces were also cultured as explants, upon murine laminin (Sigma; 1 mg/cm²) according to described methods (Goldman et al., 1992b).

Media

We used a base medium that we had earlier found permissive for neurogenesis in adult avian explant cultures (Goldman et al., 1992), modified in that the base of Dulbecco's modified Eagle's medium/Ham's F-12 was prepared without either phenol red or glutamate, and the nonessential amino acid supplement also excluded glutamate and aspartate. In cultures of dissociated avian SZ, this medium supported neurogenesis with as little as 0.625% fetal bovine serum (FBS) (unpublished observation), although the present study reports only cultures raised at a serum concentration of 10%. Dissociated samples were pelleted and resuspended in this medium, and then plated as monolayer cultures. The medium was supplemented with 10% FBS, and dialyzed to MW 1000 Da. Selected cultures were also supplemented with EGF (Collaborative Research; 20 ng/ml). All cultures were given a complete change of media after 6 d *in vitro* (DIV), with half-volume changes twice weekly thereafter.

Immunocytochemistry

Cultures were incubated for 7–28 d and fixed, and each was probed with antibodies directed against one or two neuronal antigens, which included microtubule-associated protein-2 (MAP-2; Bernhardt and Matus, 1985), neurofilament (NF; Bignami et al., 1980), N-CAM (Edelman, 1984), and MAP-5 (Huber and Matus, 1984). Cultures were also probed with antibodies directed against a variety of astrocytic (glial fibrillary acidic protein, GFAP), oligodendrocytic (O1, O4), prooligodendrocytic (A2B5 and G_{D3}, as well as O4), and microglial (CD68) antigens, to differentiate among glia (Kelly et al.,1988; Vaysse and Goldman, 1990).

Neurons were defined as those cells with typical multipolar morphology and immunoreactivity for N-CAM, MAP-2, NF, or MAP-5, but not GFAP. We used the following antibodies for neuronal identification in this study: mouse anti-N-CAM IgG (1:25; Sigma, clone OB11); rabbit anti-neurofilament serum (1:100; Dr. D. Dahl); rabbit anti-MAP-2 (1:100; Dr. I. Fisher) (Fisher et al., 1987); mouse anti-MAP-5 (1:100; Sigma, clone AA6). The protocol used for detecting each of these antigens was as previously described for MAP-2 (Goldman, 1990), with secondary antibodies appropriate to the species and idiotype of each primary antibody.

Glial cell types were characterized on the basis of previously defined criteria (Cameron and Rakic, 1991). Oligodendrocytes were defined as immunoreactive for the O4 antigen (Bansal et al., 1989), whether GFAP⁺ or GFAP⁻. Astrocytes were identified by their expression of GFAP, except for those that coexpressed GFAP and O4, which were classified as oligodendrocytic. Microglia were characterized by the CD68 antigen (Kelly et al., 1988), and pre-GFAP and/or O4 glia as either A2B5 (Eisenbarth et al., 1979) or G_{D3} immunoreactive (Goldman et al., 1984). To identify these glial types, we used the following antibodies: mouse monoclonal antibodies O4 and O1 IgM (1:50; Dr. R. Bansal), mouse anti-G_{D3} IgG (1:25; clone R24, Dr. J. Goldman), mouse A2B5 IgM (1:14; Dr. K. Fields), anti-GFAP IgG (1:100; Sigma, clone GA5), and mouse anti-microglial CD68 IgG (1:100; Dako, clone EBM11). The protocols used for each of these probes were also as previously described (Kelly et al., 1988; Vaysse and Goldman, 1990; Bansal et al., 1989). All surface antigens (O4, O1, G_{D3}, A2B5, CD68) were probed on live cells, which were fixed after antibody exposure for 10 min with cold, 2% paraformaldehyde. Skeletal antigens were probed in 4% paraformaldehyde-fixed, saponin-permeabilized cultures. All antigens were then detected using fluorescent secondary antibodies at 1:50–100.

We verified the immunoreactivity and optimal titer of each antibody in a series of embryonic rat forebrain cultures (data not shown). A separate set of control cultures, prepared to assess nonspecific immunostaining, were exposed to either mouse IgG (10 mg/ml; Sigma), mouse anti-agrin IgG (1:100; Dr. E. Godfrey), or normal rabbit serum (1:100; GIBCO), followed by appropriate secondary antibodies. None of these controls displayed significant immunostaining.

³H-Thymidine Labeling

The uptake of ³H-thymidine by antigenically defined neurons was used as an index of antecedent precursor cell mitosis *in vitro*. ³H-thymidine (0.2 μCi/plate, from 1 mCi/ml stock; 5 Ci/mM, Amersham) was added 6 hr after culture preparation, so that S-phase initiation of labeled cells would have occurred *in vitro*. Cultures were exposed to ³H-thymidine during their first 6 DIV, after which a complete medium exchange removed residual isotope. As noted, all cultures were fixed 7–28 d after establishment, and then immunostained and autoradiographed. Autoradiography was performed as previously described (Goldman, 1990; Goldman et al., 1992b), after which the percentage of ³H-thymidine⁺ neurons in each culture was calculated, and used as an index of *in vitro* neurogenesis. ³H-thymidine–labeled cells were defined as having ≥10 silver grains over their nuclei (background averaged <1 grain/10² μm²). Labeled cells were presumed to have been in S-phase at the time of ³H-thymidine exposure, and to have arisen by the *in vitro* mitosis of parental progenitors.

Calcium Imaging

Cells were challenged with a depolarizing stimulus of 60 mM K^+, during which their cytosolic calcium levels were observed. To this end, cultures were loaded with 10 μM fluo-3 acetomethoxyester (fluo-3 AM; Molecular Probes) for 1 hr at 37°C. A Bio-Rad MRC600 confocal scanning microscope, coupled to Olympus IMT-2 inverted microscope, was used to image the fluo-3 signal. Excitation was provided by the 488 nm line of a 25 mW argon laser, filtered to ≤0.1% by neutral density filters. Emission was long-pass filtered (515 nm) and detected with the confocal set to its maximal aperture (7 mm). Images were acquired every 1–5 sec and recorded on a Panasonic TQ-2028F optical disk

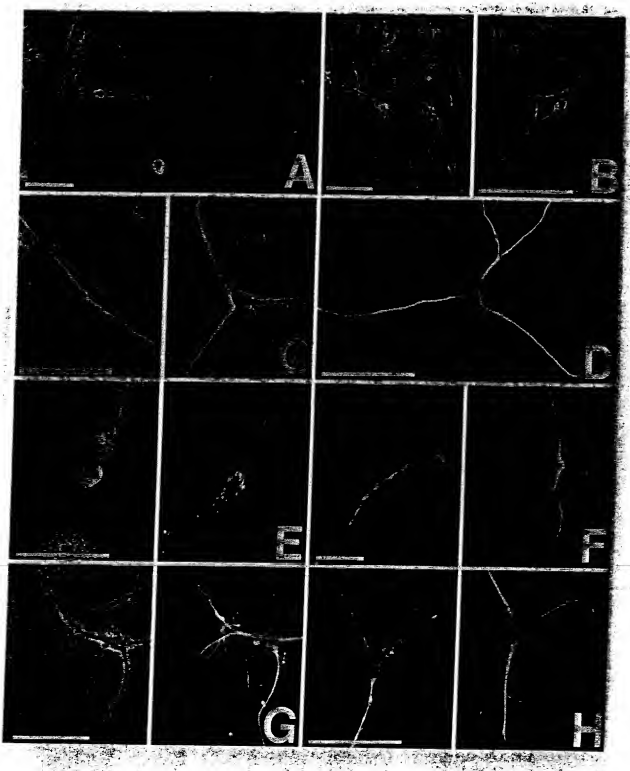

Figure 2. Neuronal differentiation in cultures of adult human temporal lobe. Neurons were found in small numbers in both explant outgrowths and dissociates of adult human SZ, and rarely, subcortical white matter. *A* and *B* display explant outgrowths derived from temporal horn SZ, in which presumptive neurons are seen upon a layer of flat astrocytes after 19 *d in vitro* (DIV). *C* shows two relatively immature cells obtained from dissociated temporal SZ, fixed and stained for MAP-2 after 8 DIV. *D* shows another MAP-2+ neuron, found in a subcortical dissociate after 18 DIV. This sample may have harbored residual SZ, but our data allow the possibility that rare precursors with neuronal potential persist in the subcortical white matter, as well as the SZ. *E* shows an N-CAM+ cell found in another culture of dissociated temporal subcortex, at 18 DIV. Small N-CAM+ cells like these were common in subcortical dissociates. The vast majority developed into O4+/N-CAM- pro-oligodendrocytes, but a small number retained N-CAM expression, failed to develop oligodendrocytic antigenicity, and instead developed neuronal morphology and antigenicity. *F* shows two such N-CAM+ cells, found in a subcortical dissociate after 18 DIV. *G* shows phase and fluorescent images of an NF+ neuron, found in an SZ dissociate after 18 DIV, while *H* displays two images of MAP-5+ cells after 15 DIV. Scale bars, 50 μm.

A
B
C
D
E
F
G

recorder. Relative changes in fluorescence were calculated and normalized against baseline fluorescence by $\Delta F/F$ (Connor et al., 1987), and background counts were subtracted from all experiments. Each experiment was carried out at 25°C in HBSS, with 60 mM K^+ exchanged for 60 mM Na^+ in the potassium-depolarizing solution.

Results

Antigenic Identification

Among the 11 brain samples, eight included ventriculotomy and hence SZ tissue. One set of cultures failed technically, and one set was lost during processing, giving us six sets of SZ cultures. Fiber-projecting, neuron-like cells were found in both SZ explant outgrowths and dissociates. These cells were typically phase bright at low power, and phase dark at higher magnification. Unlike cocultured astrocytes, these cells had no large cytoplasmic inclusions, their cell bodies lacked lamellopodial extensions onto the substrate, and they had only two or three primary processes, which ramified distally (Fig. 2). Representative examples of these neuron-like cells were found to express MAP-2, MAP-5, N-CAM, or NF, while failing to stain concurrently for GFAP. Indeed, MAP-2, MAP-5, N-CAM, and/or NF^+ neuron-like cells were each found in cultures taken from at least three of the six SZ-containing samples (Fig. 2). (Not all antigens were sought in plates derived from each SZ sample.) The antigenicity of these cells suggested that they were in fact phenotypic neurons.

Subependymal Zone Derivatives

We examined the postulate that these neurons arose from SZ progenitors, by assessing neuronal outgrowth from explants taken from the temporal SZ (a region that in the adult human is sparse in neurons), relative to that observed in temporal subcortical and cortical explants. Among six brains from which temporal SZ explants were prepared, four produced cellular outgrowth and three displayed morphologically neuron-like cells migrating upon subjacent astrocytes and/or flat glioblasts (Fig. 2). Representative explants from these brains were stained for MAP-2, MAP-5, or N-CAM; explants from one were also subjected to physiological analysis (see below). While neurons were found in the outgrowth from SZ explants, as well as in SZ dissociates (Fig. 2), no similar outgrowth was observed from a total of over 300 explants taken from the temporal neocortex (seven tissue samples, each yielding 24–36 explants dispersed among four to six culture plates) and subcortex (four samples, again yielding 24–36 explants each). Unfortunately, meaningful quantification of neuronal outgrowth from SZ explants was hindered by the variability in the amount and location of SZ obtained from each brain, which was dictated by the surgical procedure. A likely source of additional variability may have been the heterogeneous distribution of potential precursor cells within the SZ (Luskin, 1993).

Subcortical Phenotypes

In subcortical dissociates, in contrast to subcortical explants, rare neurons were noted in plates derived from two of the tissue samples, although no subcortical culture had more than 10 (as in the SZ samples, neurons were characterized antigenically as either $MAP\text{-}2^+/GFAP^-$, $MAP\text{-}5^+/GFAP^-$, or $N\text{-}CAM^+$; see Fig. 2). Not surprisingly, more fiber-bearing cells were harvested from subcortical white matter than from SZ, as a result of the white matter's enrichment in $O4^+$ oligodendrocytes and fibrous astrocytes. Since our subcortical cultures generally contained at least 10^3 fiber-bearing cells/plate, when neurons were found, they constituted <1% of the fiber-projecting cell population. Far more common were fibrous astrocytes and oligodendrocytes, which were variably $GFAP^+/O4^-$ and $GFAP^\pm/O4^+$, respectively. Astrocytes were ubiquitous and pleomorphic (Fig. 3*C–F*), but the predominant (>80%) fiber-bearing cell of the subcortical dissociates was the $O4^+$ oligodendrocyte (Fig. 3*G*); the latter was only rarely noted in the SZ dissociates and explants. Both $GFAP^+$ and $GFAP^-$ examples of $O4^+$ cells were noted.

Most of the cells found in these dissociate cultures were not fiber bearing at all, and were readily distinguished from neurons. These cells typically comprised the majority of each culture even after 6 DIV, although their relative proportions varied as a function of the culture conditions and region sampled (data not shown). These cells included capillary endothelia, flat $GFAP^-$ cells of uncertain phenotype, small and undeveloped $GFAP^+$ astrocytes, $EBM11/CD68^+$ amoeboid

←

Figure 3. Glial phenotypes. Glia were pleomorphic in these cultures, but remained identifiable on the basis of their GFAP and/or O4 expression, lack of MAP-2 and NF immunoreactivity, minimal and/or transient MAP-5 and N-CAM expression (by oligodendrocytes only), and limited calcium response to high K^+. Both SZ and cortical dissociates were composed primarily of amoeboid microglia, endothelia, flat astroblasts, and $GFAP^-$ astrocytes. In *A*, the presence of immature glia or their precursors was suggested by the presence of G_{2a}^- cells; a single G_{2a}^+ is shown here, among other G_{2a}^- glia and microglia, after 12 DIV. *B* shows corresponding phase and fluorescent views of microglia found with rare fiber-bearing cells in an SZ culture after 7 DIV. This culture was immunostained for microglial CD68 with monoclonal antibody EBM11. *C* shows an example of a mature astrocyte in an SZ culture after 27 DIV. Cultures of dissociated subcortex yielded $GFAP^+$ cells of a different morphology, with branching arbors of thin fibers, likely fibrous astrocytes. *D* and *E* show two subcortical astrocytes stained for GFAP, at 18 DIV; these cells were of strikingly "neuron-like" morphology. Some (e.g., *E, inset*) incorporated ^3H-thymidine, and were presumably generated *in vitro*. Subcortical cultures also harbored fiber-bearing cells of oligodendrocytic lineage. *F* shows the many fiber-bearing cells of a subcortical dissociate at 22 DIV. The majority were $GFAP^-$, as in *F*, in which only three $GFAP^-$ cells are seen among a field of smaller, process-bearing cells. Although these $GFAP^-$ subcortical cells included rare $MAP\text{-}2^+/MAP\text{-}5^+$ neurons (see Figs. 2, 4), most expressed the oligodendrocytic lineage marker O4. *G* shows $O4^+$ oligodendroglia (Texas *red* fluorescence) found in a subcortical dissociate after 14 DIV, with admixed $GFAP^+$ astrocytes (*green* fluorescence). The antigenic phenotype of the $O4^+$ cells ($O4^+/J1^+/A2B5^-/G_{2a}^-/MAP\text{-}2^-/MAP\text{-}5^{low-+}$) was consistent with that of pro-oligodendrocytes (Armstrong et al., 1992). These may have arisen from dedifferentiated oligodendrocytes, or from postmitotic oligodendroglial precursors. Scale bars, 50 μm.

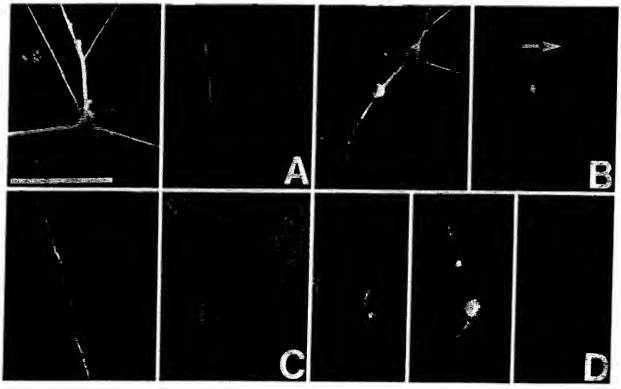

Figure 4. Neurogenesis *in vitro*. *A–C* show MAP-5$^+$ cells observed in dissociate cultures, after 15 DIV. *A* and *B* were derived from subventricular white matter, and *C* from nominal SZ; all plates were fixed after 15 DIV. The indicated cells (*arrows*) have each incorporated ^{3}H-thymidine *in vitro*, suggesting their origin from precursor mitosis. *D* shows a pair of adherent MAP-2$^+$ cells found in an SZ dissociate at 14 DIV, only one of which (*arrow*) has incorporated ^{3}H-thymidine. Scale bar, 50 μm.

and ramified microglia (Fig. 3*E*), and rare (≤10 cells/plate) A2B5$^+$ and $G_{0/1}^+$ cells (Fig. 3*A*).

Neurogenesis

Among antigenically defined neurons (MAP-2$^+$, MAP-5$^+$, N-CAM$^+$, or NF$^+$), autoradiography revealed ^{3}H-thymidine$^+$ cells in samples derived from three brains, indicating the origin of these cells from precursor mitosis *in vitro* (Fig. 4). Examples of ^{3}H-thymidine$^+$ cells expressing each of these neuronal antigens were found. However, the overall yield of ^{3}H-thymidine$^+$ neurons was low: in one representative sample of dissociated SZ, of 2×10^5 cells plated into each of three petri dishes, a total of <500 fiber-bearing cells (<0.25% of the initial cell sample) were found in the three plates after 14 DIV. The vast majority of the initial cell sample had died, and as noted above, the survivors included a mixture of flat uncharacterized cells, small stellate astrocytes, capillary endothelial cells, and microglia. Among the SZ-derived, fiber-bearing cells of neuron-like morphology, 393 were actually GFAP$^+$, while 56 were MAP-2$^+$. Of the 393 GFAP$^+$ cells, 79 (20%) were ^{3}H-thymidine$^+$, while of the 56 antigenically verified neurons, only six (11%) had incorporated ^{3}H-thymidine$^+$ *in vitro*.

Gliogenesis

^{3}H-thymidine-incorporating GFAP$^+$ astrocytes were frequently noted in cultures of all three sampled regions, including SZ, subcortex, and cortex (e.g., Fig. 3*E*). Similarly, ^{3}H-thymidine$^+$ microglia were frequently noted (data not shown). In contrast, two mitotically distinct classes of O4$^+$ cells were noted. The first was characterized by relatively ovoid, 8–10 μm cell bodies that projected a variable number (two to nine) of relatively thin, short fibers, which frequently branched within <100 μm of the soma. These cells were intensely O4$^+$, and variably GFAP$^+$ (Fig. 3*G*). These O4$^+$/GFAP$^\pm$ cells were mitotically quiescent; among a sample of 8044 such cells, culled from four plates of subcortical white matter (2011 ± 858.6 O4$^+$ cells/plate, mean ± SD), *none* incorporated ^{3}H-thymidine *in vitro*, despite the frequent observation of ^{3}H-thymidine-labeled astrocytes in the same plates. These cells likely correspond to the pro-oligodendrocytes previously characterized by Armstrong et al. (1992). In contrast, a second, comparatively uncommon category of O4$^+$ cells was characterized by a larger (15–25 μm), flatter, and more substrate-apposed soma; each cell projected several relatively thick, long and tapering, unbranched processes. These cells constituted <1% of the O4$^+$ population, and frequently incorporated ^{3}H-thymidine. The ontogeny and fate of these O4$^+$/^{3}H-thymidine$^+$ cells are now being evaluated separately (Kirschenbaum and Goldman, unpublished observation).

Cell-Specific Calcium Responses

To assess the functional capability of observed neurons, 24 cultures were loaded with the calcium-sensi-

tive dye fluo-3, and exposed to 60 mM K^+ during confocal microscopy, to detect depolarization-induced Ca^{2+} increments (Fig. 5). Of these cultures, five were derived from SZ explants, and the remainder from either subcortical (n = 15) or cortical cultures (n = 4). Glial responses to 60 mM K^+ were minimal; of a pooled sample of subcortical and cortical glia (selected for fiber-bearing astrocytes and pro-oligodendrocytes), an average increment of 25 ± 3.5% (mean ± SE, n = 83) was noted in cytosolic calcium following K^+ stimulation. In contrast, neurons displayed a rapid and reversible four-fold elevation in cytosolic calcium signal in response to K^+ (402 ± 107.0%, n = 5; $p <$ 0.0001), consistent with the expected activity of neuronal voltage-gated calcium channels (Connor et al., 1987; Hockberger et al., 1987).

Discussion

The present results suggest that the adult human forebrain harbors precursor cells that retain the potential for neuronal production and differentiation *in vitro*. These cells appear to reside predominantly in the SZ, and in this study were found in samples derived from the ventrolateral aspect of the anterior temporal horn of the lateral ventricle. The adjacent subventricular white matter was found to harbor glial precursors, in that ^{3}H-thymidine$^+$ examples of both $GFAP^+/O4^-$ and $GFAP^+/O4^+$ cells were identified, as well as a distinct population of postmitotic, $O4^+/GFAP^-$ fibrous cells similar to those described as pro-oligodendrocytes (Armstrong et al., 1992).

Limitations of Antigenic Analysis

Any antigenic determination of phenotype among brain cells is limited by the lack of absolute cell-type specificity of currently identified neuroectodermal antigens. In particular, several antigenic markers previously considered prototypic of neuronal phenotype are actually expressed in developmentally restricted time windows by glia as well as neurons. Thus, whereas MAP-2 and MAP-5 are typically characterized as neuronal proteins (Huber and Matus, 1984; Bernhardt and Matus, 1985), reactive white matter astrocytes have been shown to express MAP-2 transiently (Geisert et al., 1990), while MAP-5 can be expressed by $O4^+$ pro-oligodendrocytes in culture (Vouyiouklis and Brophy, 1993). [In this regard, we found that MAP-5 expression by $O4^+$ pro-oligodendrocytes, although generally present, was far less than, and readily distinguished from, that demonstrated by $MAP\text{-}5^+/O4^-$ cells in human SZ and subcortical cultures; similarly, oligodendrocytic MAP-5 immunoreactivity was far less than that displayed by cocultured neurons in rat forebrain control cultures (data not shown).] Like MAP-5, N-CAM is expressed by early oligodendrocytes as well as by neurons (Bhat and Silberberg, 1986), and neurofilament may also be transiently expressed by astrocytes in culture (Galileo and Linser, 1992). Thus, no one of these markers is alone sufficient to define neuronal phenotype. As a result, we used a panel of neuron-selective antigens for neuronal identification in these cultures. Although individual cultures were typically probed with only one or two antibodies, matched cultures were probed for alternative neuronal antigens; most plates were probed concurrently with anti-GFAP, or in some cases O4. We required concurrent demonstration of positive neuronal antigenicity and negative glial staining for defining neuronal identity. In addition, in selected cultures of both explanted SZ and dissociated subventricular white matter, we obtained antigen-independent verification of neuronal presence and function, by assessing the cytosolic calcium responses to depolarizing stimuli of individual fiber-bearing cells.

Physiological Characterization

Although K^+-induced calcium responses have been described among cortical astrocytes in culture (MacVicar et al., 1991; Fatatis and Russell, 1992), depolarization-induced calcium increments in glia have been of lesser magnitude than those noted in neurons under analogous conditions (Connor et al., 1987; Hockberger et al., 1987). Indeed, only under conditions of cAMP stimulation, using cultures of confluent astrocytes raised over a month *in vitro*, have astrocytes been reported to display significant K^+-evoked Ca^{2+} increments (MacVicar et al., 1991). In that same study, astrocytes raised under conditions more analogous to those of our present study; that is, subconfluent cells maintained for shorter periods of time *in vitro*, and not exposed to exogenous cAMP, displayed only minor elevations, and often decrements, in K^+-evoked cytosolic Ca^{2+}. Nevertheless, no previous study of which we are aware has directly compared the depolarization-induced Ca^{2+} responses of astrocytes, oligodendrocytes, and neurons in single cultures.

In order to control for the possibility of some cultured astrocytes displaying neuron-like Ca^{2+} responses, we exposed mixed neuronal and glial cultures of embryonic (E16) rat forebrain, after 18 DIV, to 60 mM K^+ and correlated their Ca^{2+} responses with post hoc immunocytochemistry for either MAP-2 or GFAP (Nedergaard and Goldman, unpublished observation). These cells were raised under the same conditions as their adult human brain counterparts. We found that a sample of 86 antigenically verified $MAP\text{-}2^+$ neurons, randomly sampled from five cultures, exhibited a mean Ca^{2+} increment of 267 ± 106% (mean ± SD). In contrast, a cocultured population of 126 $GFAP^+$ astrocytes displayed a mean Ca^{2+} elevation of 0.8 ± 17% (the low mean increment reflected the many astrocytes whose Ca^{2+} levels fell, by as much as 50%, in response to K^+). The response ranges of these two rat brain cell types were nonoverlapping, and the difference between them significant to $p < 0.001$. These data suggest the validity of the physiological criteria used for cell-type identification in our adult human brain cultures. Nonetheless, the response characteristics of adult cells may differ from their embryonic counterparts. Thus, even though the K^+-induced Ca^{2+} responses of cells such as that displayed in Figure 5 are consistent with neuronal phenotype, single-cell recording will be needed to establish whether these cells meet a more stringent criterion of neuronal func-

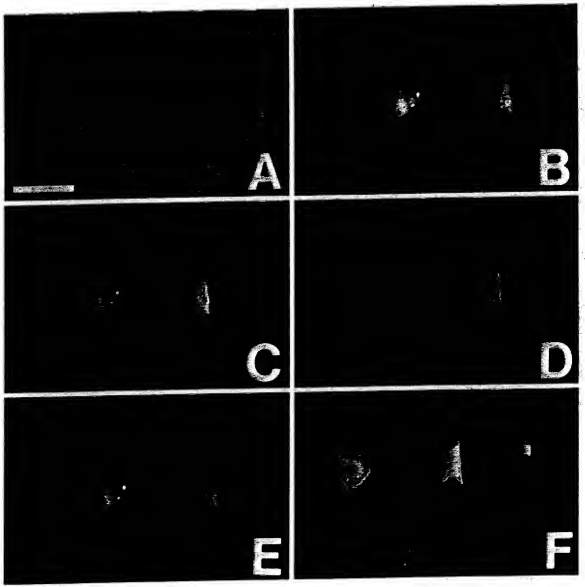

Figure 6. Calcium increments to depolarization by neurons in temporal SZ explants. Selected cultures were challenged with high K^+, to seek evidence of neuron-like depolarization-induced increments in cytosolic calcium ($[Ca^{2+}]_i$). In this plate, a temporal SZ culture was tested after 28 DIV, after loading with the Ca^{2+}-sensitive dye fluo-3. *A* shows a phase micrograph of two adjacent cells, one neuron-like and the other astrocytic. *B* shows their baseline levels of Ca^{2+}, as viewed by confocal microscopy with laser scanning at 488 nm. *C* shows the same two cells within seconds after exposure to 60 mM K^+. The neuron-like cell increased its Ca^{2+}, rapidly and reversibly, in contrast to the cocultured astrocytes. In *D*, with the addition of tetrodotoxin (TTX; 1 μM; Sigma), K^+ stimulation yielded a greater than sixfold rise in neuronal cytosolic Ca^{2+}, while astrocytic Ca^{2+}, increased less than twofold. The depolarization-induced Ca^{2+} increment of this cell suggested its neuronal phenotype, as did the TTX accentuation of its Ca^{2+}, response; the increased density of TTX-sensitive Na^+ channels in neurons, relative to glia, would have been expected to yield a neuron-selective enhancement of the K^+-stimulated Ca^{2+}, response by TTX (Ritchie and Rogart, 1977; Howe and Ritchie, 1990). (The TTX accentuation of the K^+-stimulated Ca^{2+} increment may also result from TTX's inhibition of depolarization-induced Na^+ influx; in the presence of TTX, when neuronal voltage-activated calcium channels open in response to K^+ stimulation, the Ca^{2+} cation can follow the ambient electrochemical gradient, unopposed by concurrent Na^+ entry.) In *E*, upon withdrawal of K^+ from the medium, each cell returned to its resting Ca^{2+}, level. *F* was taken after addition of the calcium ionophore Iasalocid (50 mM; Sigma), added as a positive control in order to maximize Ca^{2+} entry in both cells. These results suggested the activity of voltage-gated calcium channels in the adult-derived neurons. Scale bar, 25 μm.

tion, that of firing repetitive action potentials (Goldman and Nedergaard, 1992).

Source of the Neuronal Precursor Cells

At least some of the neuronal precursor cells reside within the SZ of the temporal horn of the lateral ventricle. These cells may represent vestiges of the embryonic SZ neuroepithelium, which retain the capacity for neuronal differentiation, and to some extent mitotic neurogenesis, when removed into tissue culture.

More difficult to explain were the rare neurons found in subcortical dissociates. These were so infrequent as to be of unclear significance; most likely, they derived from SZ inadvertently admitted into the subcortical sample during dissection. Alternatively, these neurons arose from ependymal/subependymal rests lying ectopically in the subventricular white matter

(Larroche, 1977). Similarly, since the surgical samples taken often included the parahippocampal and hippocampal gyri, these may have been included inadvertently in the subcortical dissections. In particular, any admixed hippocampal tissue would include the fascia dentata, whose granule neurons have been shown to undergo persistent turnover in adult rodents (Altman and Das, 1966; Kaplan and Hinds, 1977). Thus, some or all of the ^{3}H-thymidine$^+$ neurons found in the subcortical dissociates may have derived from mitotic dentate neuroblasts, rather than from contaminating SZ.

The observed neurons, both of our SZ and subcortical samples, might also have derived from clusters of granule neurons that lie ectopically in the basal forebrain subcortex. These cells, designated islands of Calleja, have been described across mammalian genera, including humans, and have been typically associated with subcortical olfactory pathways (Meyer et al., 1989). Interestingly, two studies have recently reported a population of newly generated neurons in the postnatal rodent basal forebrain, which migrates along a spatially restricted pathway to the olfactory bulb (Corotto et al., 1993; Luskin, 1993). It is possible that the seemingly ectopic aggregations of granule neurons in the adult human forebrain are homologous to this novel population of rodent forebrain neurons, and that our subcortical dissections may have included neuronal precursors lying within this pathway. Finally, the subcortically derived neurons might be the progeny of oligodendrocytic precursors resident within the white matter, which might retain the potential to generate neurons; such a bipotential neuronal–oligodendrocyte precursor has already been described in the embryonic rodent brain (Williams et al., 1991; Vescovi et al., 1993), although no counterpart has yet been identified in adulthood.

Whatever their geographic and cellular sources, most of the neurons identified in these cultures were unlabeled by ^{3}H-thymidine. These cells may have derived from the neuronal differentiation of precursors rendered postmitotic by the culture conditions. Alternitively, if the neurons identified in these cultures had simply represented resident neurons that had survived dissociation or explantation and regenerated neurites *in vitro*, then one might suppose that the neocortical cultures, which harbored by far the greatest number, density, and variety of neuronal phenotypes, would have yielded the most neurons. To the contrary, however, antigenically defined neurons were absent in our cortical explant outgrowths, which typically yielded only astrocytes and microglia, and rare O4$^+$ oligodendrocytes. Instead, the neurons identified in these cultures were limited to SZ and some periventricular white matter samples, precisely those regions in which neurons are scarce *in vivo*.

In our similar prior study of adult avian brain cultures raised in high-serum (Goldman, 1990), only those explants derived from neurogenic regions of the SZ displayed neuronal outgrowth, while striatal parenchymal explants failed to do so. Furthermore, prelabeling the mitotic precursor population with ^{3}H-thymidine *in vivo* revealed that most, if not all, of the neurons in these outgrowths were newly generated. Similar findings were obtained in explant cultures of the adult mouse (Lois and Alvarez-Buylla, 1993) and rat brains (Goldman and Kirschenbaum, 1994). In these rodent preparations, neuronal outgrowth was limited to explants derived from the SZ, and the emigrating neurons could be prelabeled with ^{3}H-thymidine given *in vivo*, prior to sacrifice. Thus, in both the adult songbird and rodent preparations, explant cultures selected precisely for those newly generated, migratory neurons that had just arisen from mitotic precursors.

Our present study was limited by our inability to prelabel the mitotic cells of the adult human brain prior to tissue resection and culture. Nonetheless, on the basis of (1) these parallel studies in other species, (2) the restriction of adult human neuronal outgrowth in the present study to SZ and peri-SZ subcortical explants, (3) the complete absence of neuronal outgrowth from the human cortical explants that we prepared, and (4) the occasional neurons found in human SZ and subcortical cultures exposed to ^{3}H-thymidine *in vitro*, it is likely that the ^{3}H-thymidine$^-$ neurons of these cultures were not just resident neurons that survived *ex vivo*. Rather, they appear to have derived from precursors that embarked upon neuronal differentiation *in vitro*, once removed from the tissue environment.

These precursors may have been mitotically competent *in vivo*, but lacked mitotic stimulation *in vitro*, or indeed may have been actively removed from the cell cycle by exposure to differentiation agents in culture. Alternatively, they may have derived from a resident pool of undifferentiated but postmitotic precursor cells. Evidence for the latter has been described in cultures of the postmitotic chick tectum, in which mitotically quiescent precursor cells with neuronal potential persist, and can be induced toward terminal neuronal differentiation by local neuronal depletion (Galileo et al., 1991).

The Culture Environment

In this study, we sought to increase the likelihood of finding *any* residual neural precursors in the adult brain, by raising the cultures in a high-serum medium that would optimally support neuronal differentiation, rather than precursor proliferation. Although prior reports have demonstrated that both EGF and bFGF may stimulate the division and proliferation of neural precursors in culture (Gensburger et al., 1987; Reynolds and Weiss, 1992; Richards et al., 1992; Kilpatrick and Bartlett, 1993; Ray et al., 1993), neuronal mitogenesis in these preparations appeared to require either media containing little or no serum (Reynolds and Weiss, 1992), or media supplemented with mitogen (bFGF; Richards et al., 1992). In contrast, neuronal *differentiation* from adult-derived precursor cells has been most robust in cultures raised at relatively high serum levels, in the absence of exogenous mitogenic agents, in both rodent (Lois and Alvarez-Buylla, 1993) and avian (Goldman, 1990) preparations. Indeed, when the effect of serum concentration upon adult neuronal mi-

togenesis was tested directly in explant cultures of adult finch forebrain, the neuronal ^{3}H-thymidine labeling index was found to be inversely proportional to the ambient serum level (Goldman et al., 1992b). Not surprisingly then, in the presence of the relatively high serum concentration used in this study (10%), no specific induction of neuronal mitogenesis by EGF was noted. Yet, the relatively selective detection of neurons in the SZ dissociates and explant outgrowths, and the incorporation by some of these cells of ^{3}H-thymidine, strongly suggested the existence of precursor cells with neuronal potential in these cultures. To the extent that these cells are premitotic, one might predict that they should be passageable for serial propagation. Although conditions permissive for the serial passage of embryonic rodent neural precursor cells have recently been described (Reynolds and Weiss, 1992; Kilpatrick and Bartlett, 1993; Ray et al., 1993), conditions appropriate for the sequential clonal expansion and neuronal differentiation of adult human precursor cells remain to be defined.

Lineage Potential of Single Precursor Cells

The presence of ^{3}H-thymidine$^+$ neurons and glia in these cultures raises the possibility that both are arising from a common precursor cell. Multipotential precursors have been demonstrated *in vivo* during the development of both the retina (Turner and Cepko, 1987; Wetts and Fraser, 1988) and intracortical forebrain, including the neostriata of both mammals (Temple, 1989; Halliday and Cepko, 1992) and birds (Galileo et al., 1990; Gray and Sanes, 1992). In the avian neostriatum, these precursors may remain pluripotential even into adulthood (Goldman et al., 1992a). In contrast, in mammalian *neocortical* ontogeny, committed lines of neuronal and glial SZ precursor cells become established prenatally (Levitt et al., 1981, 1983; Walsh and Cepko, 1988; Luskin et al., 1993), while distinct lines of oligodendrocytic and astrocytic precursors evolve thereafter (LeVine and Goldman, 1988).

Even postnatally, however, some rodent SZ precursor cells remain bipotential for oligodendrocytic and astrocytic production (Levison and Goldman, 1993). Indeed, during late embryonic development, both phenotypically committed and pluripotent precursors may coexist in the telencephalic ventricular zone; a bipotential cortical neuronal–oligodendrocytic precursor is present in the E16 rat forebrain (Williams et al., 1991), as are lineage-restricted neuronal, astrocytic and oligodendrocytic progenitors (Price and Thurlow, 1988; Price et al., 1991). Unfortunately, the present data do not allow us to distinguish whether the precursor cells of the adult human brain are committed to restricted phenotypes, or whether they are pluripotential for neurons and glia. As an additional consideration, the culture environment might determine not only the mitotic activity, but also the lineage commitment of these precursors; their phenotypic potential *in vitro* might differ from that realized *in vivo*. Thus, the ability of these precursors to produce neurons in culture does not necessarily suggest their capacity to do so *in vivo*.

The Natural History of Adult Primate SZ Precursor Cells

Given the retention of neuronal precursor cells in adulthood, the absence of neurogenesis in the mature primate brain (Rakic, 1985; Eckenhoff and Rakic, 1988) is intriguing. Although the adult primate appears to exhibit little if any forebrain neurogenesis *in situ* (Rakic, 1985; Eckenhoff and Rakic, 1988), our data suggest that once removed into culture, residual precursor cells may resume neuronal production and differentiation. Indeed, the adult rhesus monkey has been found to have "hot spots" of mitotic cells in the subependymal layer of the anterior horn of the lateral ventricle (Rakic and Kornack, 1993). This observation suggests that telencephalic SZ precursor populations may remain actively mitotic in the adult primate forebrain, but that these cells may fail to generate neurons *in vivo*. Instead, the ability of their progeny to either survive or differentiate into neurons may be compromised passively, by a lack of some rate-limiting trophic agent (Goldman and Kirschenbaum, 1994), or actively, through the tonic suppression of neurogenesis by either soluble or contact-dependent agents within the local tissue environment.

Indeed, even if the newly generated cells could be rescued *in vivo*, whether by the introduction of survival factors lacking in adult primate brain, or by the active inhibition of tonic suppressive agents, their fate and function could not yet be predicted or dictated. Regarding the SZ cells sampled in this study, their location in the inferior horn of the lateral ventricle, in the anteromedial temporal lobe, suggests that they might contribute neuronal progeny not only to the hippocampal archipallium, but also to the subjacent subiculum, parahippocampal gyrus, and entorhinal cortex, or even the adjacent amygdala and prepyriform cortex. If these daughter neurons were induced to survive *in vivo*, we yet have no way of knowing whether their phenotype would be restricted to a prespecified subset of neurons, or whether they could be recruited into any local circuit having a permissive route and substrate for migration and open synaptic space. In this regard, the population of neuronal precursors for the postnatal rat olfactory bulb, to which we referred previously (Corotto et al., 1993; Luskin, 1993), generates a phenotypically and regionally restricted cohort. Neurons generated from these SZ cells migrate to and insert within the olfactory bulb, but do not appear to contribute to other regional circuits. This suggests that these young neurons are subject either to a predetermined restriction of their fate, or a sharply demarcated distribution of environmental cues limiting their migration. Interestingly, the progenitors from which these rodent neurons arise lay in the anterobasal frontal SZ, a region phylogenetically analogous to the human anteromedial temporal SZ (Farbman, 1991), which corresponds to the SZ segment sampled in our present study.

Nonpermissiveness for Reactive Neurogenesis in vivo

In light of the occurrence of compensatory neurogenesis following brain lesion in lower vertebrates, in-

cluding fish (Anderson and Waxman, 1985), lizards (Font et al., 1991) and birds (Cheng and Zuo, 1993), the primate's apparent lack of such restorative neurogenesis is puzzling (Goldman-Rakic, 1980). *In vivo*, the precursor cells may lack either mitotic stimulation or postmitotic signals permitting neuronal differentiation or survival. Alternatively, these precursors may be tonically inhibited from generating neurons after embryogeny; their ability to generate neurons *in vitro* but not *in vivo* may reflect the suppressive effect on neurogenesis of local, tissue-derived factors. The identity of these agents in the mammalian brain remains problematic. In the adult avian brain, the tonic inhibition of neurogenesis by non-estrogenic ovarian influences (Hidalgo and Goldman, 1993) has opened the possibility that gonadally derived peptides, such as members of the inhibin-β family, might be operative in the restriction of neurogenesis in the adult avian forebrain. Indeed, the close structural homology of ovarian inhibin-β to transforming growth factor β (TGF-β) might suggest a more general role for the latter in the regulation of neurogenesis in adulthood; among olfactory precursor cells, for example, TGF-β has already been shown to potentiate neuroblastic departure from the cell cycle and terminal neuronal differentiation (Mahanthappa and Schwarting, 1993).

Teleologically, it is a reasonable assumption that the nonpermissiveness of the adult brain toward compensatory neurogenesis has adaptive value, particularly in light of the progressive restriction in the temporal and geographic extents of adult neurogenesis as one progresses in phylogeny (Rakic and Kornack, 1993). Nonetheless, whatever the basis for this phylogenetic restriction in adult neurogenesis, our data suggest that it does not stem from a lack of appropriate progenitor cells. Rather, once removed from the milieu of adult brain parenchyma, residual precursor cells can resume the production of antigenically and physiologically characteristic neuronal progeny.

Notes

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EVIDENCE APPENDIX
EXHIBIT 9

Zhou et al., "The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification," *Cell* 109:61-73 (2002)

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The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification

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Summary

OLIG1 and OLIG2 are basic-helix-loop-helix (bHLH) transcription factors expressed in the pMN domain of the spinal cord, which sequentially generates motoneurons and oligodendrocytes. In *Olig1/2* double-mutant mice, motoneurons are largely eliminated, and oligodendrocyte differentiation is abolished. Lineage tracing data suggest that *Olig1*$^{-/-}$ *2*$^{-/-}$ pMN progenitors instead generate V2 interneurons and then astrocytes. This apparent conversion likely reflects independent roles for OLIG1/2 in specifying motoneuron and oligodendrocyte fates. *Olig* genes therefore couple neuronal and glial subtype specification, unlike proneural bHLH factors that control the neuron versus glia decision. Our results suggest that in the spinal cord, *Olig* and proneural genes comprise a combinatorial code for the specification of neurons, astrocytes, and oligodendrocytes, the three fundamental cell types of the central nervous system.

Introduction

The three fundamental cell types of the vertebrate central nervous system (CNS) are neurons, astrocytes, and oligodendrocytes. This basic triad comprises many hundreds or even thousands of distinct neuronal subtypes, in addition to subtypes of astroglia and perhaps of oligodendroglia as well (Raff, 1989; Woodruff et al., 2001). The molecular mechanisms by which these diverse neural cell types are properly generated in space and time are incompletely understood. In recent years, a great deal has been learned about the transcriptional control of the neuron-glial fate decision (Tomita et al., 2000; Nieto et al., 2001; reviewed in Vetter, 2001) and about the control of neuron subtype specification (Briscoe et al., 2000; Jessell, 2000). Rather less is known, however, about the transcriptional control of glial subtype determination.

Two major classes of transcription factors have emerged as determinants of neuron versus glial fate determination and of neuron subtype specification: the basic-helix-loop-helix (bHLH) factors (Vetter, 2001) and homeodomain (HD) factors (Jessell, 2000), respectively. In vertebrates, bHLH factors homologous to the *Drosophila* proneural genes, such as the *Neurogenins (Ngns)* (Gradwohl et al., 1996; Ma et al., 1996; McCormick et al., 1996) and *Mash1* (Johnson et al., 1990), promote neuronal differentiation at the expense of the glial fate (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001b). In the spinal cord, a combinatorial code of HD transcription factors specifies the regional identity of progenitor domains along the dorso-ventral axis (Briscoe et al., 2000; Jessell, 2000). Motoneurons are generated from the pMN domain, while V0, V1, V2, and V3 interneurons are generated from the p0, p1, p2, and p3 domains, respectively (Briscoe et al., 2000; Jessell, 2000). This discontinuous patterning arises from mutually repressive interactions between the HD factors that specify adjacent progenitor domains (Briscoe et al., 2000; Muhr et al., 2001).

Recently, we and others identified a subclass of neural bHLH factors, called *Olig* genes (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). In the mouse, there are two *Olig* genes that are specifically expressed in oligodendrocyte precursors, called *Olig1* and *2* (Lu et al., 2000; Zhou et al., 2000), while in the chick a single gene orthologous to *Olig2* has been identified (Mizuguchi et al., 2001; Zhou et al., 2001). In the spinal cord, oligodendrocyte precursors emerge from a highly restricted domain of the ventral ventricular zone (Miller, 1996; Richardson et al., 2000). This region is precisely demarcated by expression of *Olig1* and *Olig2* (Lu et al., 2000; Zhou et al., 2000). *Olig2* is sufficient to cause ectopic differentiation of oligodendrocytes in the chick spinal cord when misexpressed together with the HD factor Nkx2.2 (Sun et al., 2001a; Zhou et al., 2001), while *Olig1* promotes oligodendrocyte differentiation in rodent cortex (Lu et al., 2001).

Prior to oligodendrogliogenesis, the domain of *Olig2* expression corresponds to the pMN domain, from which motoneurons are generated (Takebayashi et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001). Gain-of-function experiments suggest that OLIG2 plays a determinative role in patterning the pMN domain and also initiates motoneuron differentiation and cell cycle arrest, in part by promoting expression of *Ngn2* (Mizuguchi et al., 2001; Novitch et al., 2001). These data suggest that OLIG2 sequentially controls both motoneuron and oligodendrocyte fate determination. Interestingly, the bHLH factor appears to function in both cases as a transcriptional repressor (Novitch et al., 2001; Zhou et al., 2001).

To rigorously assess the requirement for *Olig* genes in motoneuron and oligodendrocyte differentiation, we have generated double-homozygous mice lacking both *Olig1* and *Olig2*. In *Olig1/2* double mutants, presumptive motoneuron precursors are transformed into V2 interneuron precursors, and oligodendrocytes are lost throughout the brain and spinal cord. Surprisingly, many *Olig2*-expressing oligodendrocyte precursors are transformed into astrocytes. Thus, in the absence of *Olig1/2* function, the sequential production of motoneurons and oligodendrocytes is converted into the sequential production of interneurons and astrocytes. These data suggest that *Olig* genes couple neuronal and glial subtype specification.

Results

Generation of *Olig1* and *Olig2* Double-Mutant Mice

The coexpression of *Olig1* and *Olig2* in vivo (Zhou et al., 2000) raised the possibility that deletion of either of

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these genes alone might be compensated by the function of the other. In order to circumvent this problem, we decided to generate an *Olig1* and *Olig2* double mutant. The mouse *Olig1* and *Olig2* genes are tightly linked on chromosome 16, about 36 kb from each other (data not shown). In order to preserve the *Olig1-Olig2* intergenic region, double-mutant mice were generated through two rounds of homologous recombination in ES cells. The *Olig2* coding region was replaced by a targeting cassette composed of a histone-GFP (hGFP) fusion (Kanda et al., 1998) and PGK-neomycin (Figures 1A and 1B), while the *Olig1* coding region was replaced by a tau-LacZ and PGK-hygromycin cassette (Figures 1D and 1E).

Using a Cre/lox-mediated analytic strategy (see Experimental Procedures), two ES clones were identified in which the *Olig1* and *Olig2* targeted loci lay in *cis* (Figure 1H). Sister cells from these clones, unmodified by Cre recombinase, were injected into blastocysts, and the targeted alleles were transmitted through the germ line (Figures 1C and 1F). Double-heterozygous mice were born at the expected Mendelian frequency and were viable and fertile. However, no live births of homozygous mice were observed, and starting from E18.5, the homozygous embryos appeared smaller than their littermates.

We initially examined the pattern of GFP and LacZ expression in heterozygous *Olig1*$^{+/-}$ *Olig2*$^{+/-}$ embryos between E9.5 and E16.5. At E9.5, GFP was strongly expressed in a ventral domain of the spinal cord that corresponds to pMN (Figure 1I), similar to the pattern of endogenous *Olig2* expression (Figure 1J; also see Takebayashi et al., 2000). In contrast, only a few cells were observed to be weakly lacZ positive at this stage (Figure 1N), in agreement with the relatively weak expression of endogenous *Olig1* during the period of neurogenesis (Figure 1O). At E16.0, when *Olig2* expression is restricted to oligodendrocyte precursors (Lu et al., 2000; Zhou et al., 2000), nearly all OLIG2-positive cells were also GFP-positive (Figures 1K–1M, arrows). In addition, GFP colocalized extensively with the *Olig1* knockin marker tau-lacZ (Figures 1P–1R, arrows), consistent with the coexpression of endogenous *Olig1* and *2* in oligodendrocyte precursors (Zhou et al., 2000). Thus, in heterozygous *Olig1*$^{+/-}$ *Olig2*$^{+/-}$ mice, the expression of GFP and tau-lacZ faithfully recapitulates the pattern of endogenous *Olig2* and *Olig1* expression in cells of both the motoneuron and oligodendrocyte lineages.

Deletion of *Olig2* and *Olig1* Results in Loss of Motoneurons and a Concomitant Ventral Expansion of V2 Interneurons

We first focused our analysis of *Olig1/2* double-mutant embryos on the generation of several neuronal subtypes derived from the four ventral-most progenitor domains of the spinal cord: En1^{+} V1 interneurons, Chx10^{+} V2 interneurons, Isl1/2^{+} and Hb9^{+} motoneurons, and Ngn3^{+} V3 interneurons (Figures 2A–2E). Most Isl1/2^{+}, Hb9^{+} motoneurons were lost at all axial levels of the homozygous mutant spinal cord at E10.5 (Figures 2H, 2I, and 2K, white bars), while the number of such motoneurons was the same in heterozygote and wild-type (Figures 2C, 2D, and 2K, dark and light gray bars). The loss of motoneurons in the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ spinal cord was not due to cell death, as no increased apoptosis was detected by the TUNEL assay at this stage (Figures 3O and 3T). Although very few presumptive motoneurons (Isl1/2^{+}, Hb9^{+} cells) were detectable at E10.5 in *Olig1*$^{-/-}$*Olig2*$^{-/-}$ embryos (Figures 2H and 2I, arrowheads), it was possible that a normal number of these neurons might be recovered at later stages through compensatory mechanisms. However, at E13.5, neither somatic (Figures 2L and 2M, arrows) nor visceral (Figures 2L and 2M, arrowheads) motoneurons were detected in the *Olig1*$^{-/-}$ *Olig2*$^{-/-}$ spinal cord (Figures 2P and 2Q, arrows and arrowheads, and 2T, white bars). Moreover, no projecting axons were observed in the ventral root (Figures 2O and 2S, arrows), consistent with a lack of both classes of spinal motoneurons.

In contrast to the dramatic loss of motoneurons, the number of Chx10^{+} cells, which derive from the p2 domain just dorsal to the pMN domain, was increased by about 80% in the double-null mutant spinal cord at E10.5 (Figures 2B versus 2G; 2K, Chx10, white bar). Furthermore, many Chx10^{+} cells occupied a more ventral position, in territory normally occupied by motoneurons (Figure 2G, yellow arrowhead). The increased number and ventral expansion of Chx10^{+} V2 interneurons were also apparent at E13.5 (Figures 2N, 2R, and 2T). The number and distribution of En1^{+} V1 interneurons and Ngn3^{+} V3 interneurons, by contrast, were largely unaltered in the mutant (Figures 2A, 2E, 2F, 2J, and 2K).

The preceding data suggested that in the absence of *Olig1/2* function, pMN progenitors might give rise to V2 interneurons instead of motoneurons. To confirm this, we used the *Olig2* knockin marker hGFP as a short-term lineage tracer to compare the identities of the neuronal progeny derived from the pMN domain of heterozygous versus homozygous *Olig1/2* double-mutant embryos. In heterozygotes, *Olig2-hGFP*-derived precursors gave rise to Islet1/2^{+} motoneurons (Figure 3A, yellow cells) but not Chx10^{+} V2 interneurons (Figure 3B). By contrast, in the homozygotes there were many GFP^{+}, Chx10^{+} cells present in the marginal zone lateral to the pMN domain of the ventricular zone (Figure 3G, yellow cells). At no time did we detect any Islet1/2^{+} Chx10^{+} phenotypically hybrid cells (data not shown). In the immediately overlying p2 domain, Chx10^{+} GFP^{-} V2 interneurons were produced in both heterozygotes and homozygotes (Figures 3B and 3G, white arrowheads). These data strongly suggest that precursor cells from the pMN domain of *Olig1/2* homozygous animals generate V2 interneurons instead of motoneurons.

Irx3 Is Derepressed in pMN in the Absence of *Olig2* and *Olig1* and Respecifies pMN to p2

The loss of motoneurons and concomitant ventral expansion of V2 interneurons in the double-null mutant could reflect a conversion of the pMN domain to a p2 identity. Consistent with this idea, the expression of Irx3, a p2 domain patterning molecule (Briscoe et al., 2000), expanded into the pMN domain in *Olig1*$^{-/-}$*Olig2*$^{-/-}$ double mutants at E10.5 (Figures 3C and 3H, arrows). Pax6 expression, which is high in the p2 domain but lower in the pMN domain (Figures 3D, arrow, and 3E), also

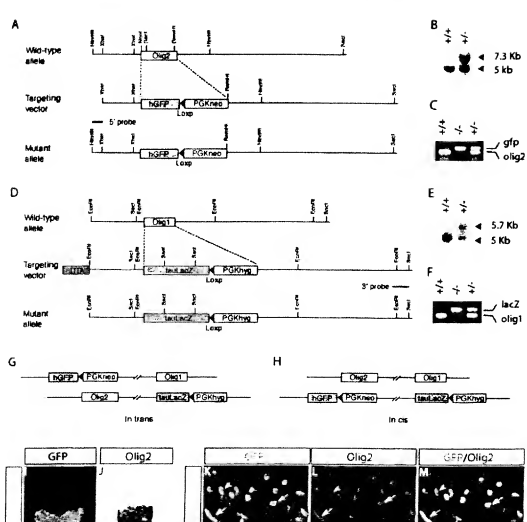

Figure 1. Inactivation of *Olig1* and *Olig2* by Homologous Recombination

(A–C) First round of homologous recombination at the *Olig2* locus.

(A) A *Histone-EGFP/loxp/PGK* neomycin cassette replaced the *Olig2* coding region.

(B) Correct recombination at *Olig2* locus verified by Southern blot analysis of ES clones.

(D–F) Second round of homologous recombination at the *Olig1* locus.

(D) A *tau-LacZ/loxp/PGK* hygromycin cassette replaced the *Olig1* coding region in ES cells in which one *Olig2* locus had been successfully targeted. Abbreviation: DTA, diphtheria toxin A chain.

(E) Correct recombination at the *Olig1* locus verified by Southern blot.

(C and F) Successful germline transmission of the targeted *Olig2* and *Olig1* alleles in *Olig1,2* double-mutant embryos confirmed by genotyping with specific PCR primers.

(G and H) Schematic diagram showing that the two targeting cassettes could lie either in *trans* (G) or in *cis* (H) to each other. A Cre/Loxp analysis was used to identify ES cells in which the two cassettes lie in *cis* (see Experimental Procedures).

(I, J, N, and O) Expression of histone-EGFP and tau-LacZ in the heterozygotes. Thoracic spinal cord sections of E9.5 *Olig1*$^{+/-}$ *Olig2*$^{+/-}$ heterozygous embryos were either labeled with anti-GFP antibody (I) or anti-LacZ antibody (N) or were probed by in situ hybridization with cRNAs against Olig2 (J) and Olig1 (O).

(K–M and P–R) Thoracic spinal cord sections from E16.0 *Olig1*$^{+/-}$ *Olig2*$^{+/-}$ heterozygous embryos were double labeled with antibodies to GFP and Olig2 (K–M) or tc GFP and lacZ (P–R). Extensive colocalization of GFP and Olig2 (K–M) as well as GFP and tau LacZ (P–R) were observed. Arrows indicate double-positive cells. A small number of GFP^{+} cells was found to be *Olig2*$^{-}$ at this stage (K–M, arrowheads).

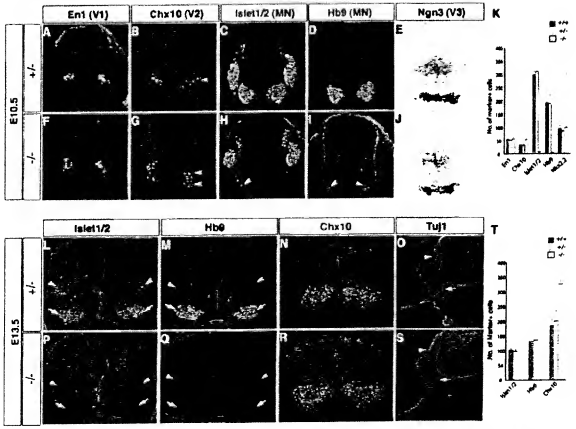

Figure 2. Loss of Isl1/2^{+}Hb9^{+} Motoneurons and Concomitant Ventral Expansion of Chx10^{+} V2 Interneurons in the Absence of *Olig1* and *Olig2*

(A–K) Crosssections of E10.5 thoracic spinal cord were stained with antibodies to detect four types of ventral neurons: En1^{+} V1 interneurons (A and F), Chx10^{+} V2 interneurons (B and G, arrowheads), Isl1/2^{+} and Hb9^{+} motoneurons (C, D, H, and I), and Ngn3^{+} V3 interneurons (E and J). Note the dramatic reduction of Isl1/2^{+} and Hb9^{+} motoneurons in the homozygous spinal cord (C, D, H, and I), while the Chx10^{+} V2 neurons increased in number and expanded ventrally (B and G, arrowheads). Arrowheads in (H) and (I) indicate the few residual motoneurons formed in the homozygote. White and yellow arrowheads in (B) and (G) indicate Chx10^{+} V2 interneurons in the p2 and pMN domains, respectively. Quantitative analysis is shown in (K). The number of marker positive cells is presented as mean ± S.D. from nine sections of three embryos. Isl1/2^{+}Hb9^{+} motoneurons decreased to <5%, and Chx10^{+} V2 neurons increased ~60% in the double-null mutant compared to heterozygote or wild-type.

(L–T) Motoneuron phenotype at E13.5. Both visceral (L and M, arrowheads) and somatic (L and M, arrows) motoneurons were lost in the homozygotes (P and Q, arrowheads and arrows). In addition, projecting axons were selectively lost in the ventral root of the null mutant (O and S, arrows). Quantitative analysis revealed a 60% increase in the number of Chx10^{+} cells in the double-null mutant (T; mean ± S.D., six sections from 2–3 animals).

increased in the pMN domain of the null mutant, so that cells in both pMN and p2 were now expressing equally high levels of Pax6 (Figure 3I, arrow). The observed ventral expansion of Irx3 is predicted by the observation that Irx3 and OLIG2 exert crossrepressive activities in gain-of-function assays (Novitch et al., 2001). Surprisingly, however, it did not cause a complete loss of GFP expression from the *Olig2* locus (Figures 3P and 3R), perhaps because the repressive effect of Irx3 is overridden by the higher levels of Shh signaling more ventrally. The expression of several other ventral spinal cord patterning molecules, including Dbx2, Nkx6.1, Nkx6.2, and Nkx2.2 (Briscoe et al., 2000), was unchanged in *Olig1/2* double mutants (data not shown). Taken together, these data suggest that in the absence of *Olig1/2*, pMN cells are converted to a p2 identity (Figures 3E and 3J).

Olig2 and *Olig1* Regulate Neurogenin 2 Expression in pMN

Ectopic expression studies in chick suggested that deletion of *Olig2* and *Olig1* should cause a loss of *Ngn2* expression in the pMN domain (Novitch et al., 2001). Consistent with this prediction, no NGN2-positive cells were detected in the presumptive pMN domain of the *Olig1*$^{-/-}$ *Olig2*$^{-/-}$ mutant at E10.0 (Figure 3P), while prominent NGN2 expression was evident in the GFP^{+} pMN domain of the heterozygous spinal cord (Figure 3K, yellow cells). The lack of apoptotic cells detected by TUNEL labeling in the mutant spinal cord (Figures 3O and 3T) suggests that the loss of NGN2^{+} cells in the pMN domain does not reflect cell death. We also observed a slight ventral expansion of MASH1 into pMN in *Olig1/2* double mutants (Figures 3L versus 3Q, arrow).

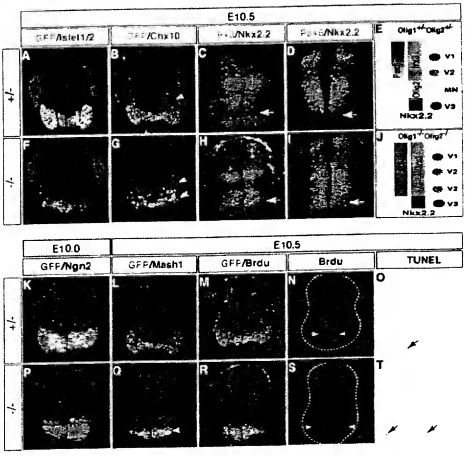

Figure 3. Derepression of *Irx3* and Repression of *Neurogenin2* in the pMN Domain of *Olig1*$^{-/-}$*Olig2*$^{-/-}$ Embryos

(A, B, F, and G) OLIG2-hGFP^{+} precursors in pMN generate Islet1/2^{+} motoneurons in heterozygotes (A, yellow cells) but instead produce Chx10^{+} V2 interneurons in homozygotes (G, yellow arrowhead). White arrowheads in (B) and (G) indicate Chx10^{+}, OLIG2-hGFP^{+} V2 interneurons generated from the p2 domain.

(C and H) Irx3 is derepressed in the pMN of the mutant spinal cord (H, arrow), as is Pax6 (D and I; arrows indicate pMN domain).

(E and J) Summary of the ventral spinal cord patterning defects in *Olig1/2* mutants.

(K, P) Neurogenin 2 (Ngn2) expression is selectively lost in pMN of the mutant at E10.0, while a delayed expansion of MASH1 into this domain is detected at E10.5 (L and Q, arrowhead).

(M, N, R, and S) Many BrdU^{+} cells persist outside the ventricular zone of the pMN in the null mutant (S, arrowheads) compared to heterozygotes (N); the same fields with GFP expression superimposed are shown in (R) and (M), respectively.

(O and T) No significant cell death was observed in the spinal cord at this stage (E10.5). Arrows indicate apoptotic cells outside the spinal cord.

As *Mash1* has recently been shown to be necessary and sufficient for Chx10 expression (Farras et al., 2002), these results may explain how V2 interneurons can differentiate from the mutant pMN despite the absence of NGN2 (Scardigli et al., 2001).

Since NGN2 has been shown to promote cell cycle exit and terminal differentiation (Farah et al., 2000; Novitch et al., 2001), we reasoned that the loss of *Ngn2* expression in pMN might cause delayed cell cycle exit by pMN-derived precursors as they migrated from the ventricular zone. To assess this, we measured BrdU incorporation after a 2 hr pulse in vivo at E10.5. An increased number of BrdU^{+} cells was detected outside the ventricular zone in the GFP^{+} region of the double-null mutant (Figures 3M, 3N, 3R, and 3S, arrowheads). These data suggest that pMN cells lacking OLIG2 fail to exit the cell cycle before migrating into the marginal zone. The presence of ectopic MASH1^{+} cells in pMN is not inconsistent with this observation, as we have recently found that MASH1 promotes cell cycle arrest less efficiently than does NGN2 (Lo et al., 2002).

Failure of Oligodendrocyte Development in *Olig1/2* Double Mutants

We next examined the phenotypic consequences of *Olig2* and *Olig1* deletion on the development of oligodendrocytes. To detect oligodendrocyte precursors, *PDGFRα* and *Sox10* were used as markers (Hall et al., 1996; Zhou et al., 2000), while *MBP* and *PLP/DM20* were used to detect mature oligodendrocytes (Zhou et al., 2001). At no time did we detect expression of any of these markers in the *Olig1/2* double-homozygous mutant at all axial levels of spinal cord examined (Figure 4, $-/-$, and data not shown) as well as in all brain

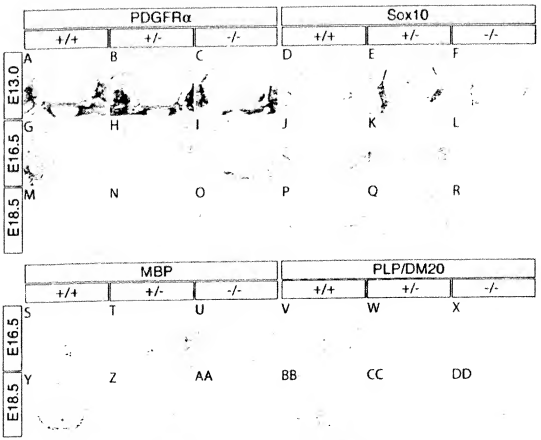

Figure 4 Spinal Cord Oligodendrocytes Fail to Develop in the Absence of *Olig1* and *Olig2*

(A–R) In situ hybridization with the oligodendrocyte precursor markers *PDGFRα* and *Sox10* on crosssections of thoracic spinal cord at indicated stages. Note the total absence of *PDGFRα* and *Sox10* expression in the null mutant spinal cord (C, F, I, L, O, and R).

(S–DD) No *MBP*$^{+}$ or *PLP/DM20*$^{+}$ mature oligodendrocytes were detected in null mutant spinal cord (U, X, AA, and DD). In addition, the number of *MBP*$^{+}$ and *PLP/DM20*$^{+}$ oligodendrocytes was smaller in the heterozygotes (Z and CC) than in wild-type (Y and BB) at E18.5.

areas examined (Figures 5D–5F and data not shown). In contrast, numerous cells expressing these oligodendrocyte precursor and differentiation markers were present in the wild-type (Figure 4, +/+) and heterozygous (Figure 4, +/-) spinal cord and brain (Figures 5A–5C and data not shown). Thus, there is a total failure of oligodendrocyte formation in the *Olig1*$^{-/-}$ *Olig2*$^{-/-}$ double mutant.

Cell counts at E16.5 revealed no decrease in the number of *PDGFRα*$^{+}$ precursors at thoracic levels of the spinal cord in heterozygotes compared to wild-type (297 ± 17 versus 290 ± 19 cells per 18 μm section, respectively, mean ± S.D., n = 6 sections from three embryos). Consistent with these data, in heterozygotes at E16.5 and P8, *MBP* and *PLP/DM20* expression was normal (Figures 4S, 4T, 4V, and 4W and data not shown). Surprisingly, however, between E18.5 and P0, there was significantly less expression of these mature oligodendrocyte markers in the heterozygotes compared to wild-type (Figures 4Y, 4Z, 4BB, and 4CC and data not shown). These data suggest that a full dosage of *Olig* genes is required for the progression of oligodendrocyte differentiation but not for the initiation of this process.

Olig1 Is Functionally Redundant with *Olig2* in Hindbrain Oligodendrocyte Development

We next examined the phenotype of *Olig1/2* double-knockout embryos in the hindbrain. As in the spinal cord, hindbrain somatic motoneuron differentiation did not occur in *Olig1/2* double mutants, as evidenced by the loss of Isl1/2^{+}, Hb9^{+} cells and of the XIIth cranial somatic motor nerve (Figures 5G–5I versus 5J–5L, arrows). In contrast, visceral motoneurons, identified by coexpression of Isl1/2 and Phox2b (Dubreuil et al., 2000), were generated (Figures 5J–5L, arrowheads). These results are consistent with the fact that visceral motoneurons in the hindbrain derive from the p3 domain (Briscoe et al., 1999), which does not express either *Olig1* or *Olig2* (data not shown).

In *Olig2*$^{-/-}$ single mutants, oligodendrocytes are spared in the hindbrain while they are lost throughout the spinal cord (Lu et al., 2002 [this issue of *Cell*]). In contrast, we found that neither oligodendrocyte precursors nor mature oligodendrocytes were generated in the hindbrain of *Olig1/2* double mutants (Figures 5D–5F), as was the case in all other brain areas examined (not shown). Taken together, these data suggest that *Olig1*

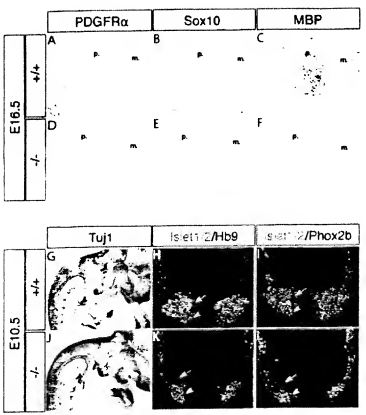

Figure 5. Loss of Hindbrain Oligodendrocytes in the Null Mutant Is Preceded by the Loss of Hindbrain Somatic But Not Visceral Motoneurons

(A–F) In situ hybridization was performed on head sagittal sections of E16.5 wild-type or double-null mutant embryos. The pictures were taken from the midbrain-hindbrain region encompassing pons (p.) and medulla (m.). No oligodendrocytes were present in the hindbrain (D–F) as well as other brain areas of the double mutants (data not shown).

(G and J) Whole-mount Tuj1 antibody labeling of E10.5 embryos. The XIIth cranial motor nerve (hypoglossal, arrowhead) was missing from the homozygote.

(H–L) In the caudal hindbrain of the null mutant, Isl1/2^{+} and Hb9^{+} somatic motoneurons were lost (K and L, arrows), while Isl1/2^{+} and Hb9^{+} visceral motoneurons were still present (K and L, arrowheads).

can compensate for the lack of *Olig2* in oligodendrocyte (but not somatic motoneuron) generation in the hindbrain but not in other regions of the CNS.

Oligodendrocyte Precursors Are Transformed into Astrocytes in the Absence of *Olig2* and *Olig1*

The absence of oligodendrocyte precursors in *Olig1/2* double-mutant embryos could reflect a failure of specification, their death, or their respecification into other cell types. To distinguish between these possibilities, we first tested whether there was increased cell death in the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ spinal cord from E12 to E14, a period when oligodendrocyte precursors are specified in the ventricular zone. TUNEL labeling detected no increase in apoptotic cells in either the ventricular zone or elsewhere in the spinal cord during this interval (data not shown). Next, we used the knockin marker hGFP as a short-term lineage tracer to compare the fate of *Olig2*-expressing progenitors in the presence or absence of *Olig1/2* function.

In heterozygous embryos at E13.5, individual GFP^{+} precursors could be seen migrating away from the focus of *Olig2* expression in the ventricular zone (Figure 6A, arrow and white arrowheads). By E16.5, only a few GFP^{+} cells remained at this focus (Figure 6B, arrow), and most had migrated into the gray matter. By E18.5, GFP^{+} oligodendrocyte precursors were evenly distributed throughout the spinal cord, and ventricular expression was no longer detected (Figure 6C). The pattern of migration of GFP^{+} cells in the heterozygous spinal cord closely resembles that revealed by antibody staining for endogenous OLIG2 protein (Figures 1I–1K, arrows and data not shown).

In homozygous mutant embryos, the distribution of GFP^{+} cells was different in several respects. First, although many GFP^{+} cells were present in the ventricular zone at E13.5 (Figure 6D, arrow), there was little migration into the gray matter. Second, the ventricular focus of GFP expression appeared larger in homozygous than in heterozygous embryos (Figures 6A versus 6D, arrows). Cell counts indicated a similar number of GFP-expressing cells in the null mutant versus heterozygous spinal cord at this stage (Figure 6G, E13.5), suggesting that *Olig2*-expressing cells may have been generated in correct numbers in the homozygote but somehow failed to migrate on schedule. At E16.5 and E18.5, however, there was a reduction in the number of GFP^{+} cells in the double mutant (Figure 6G, white bars). This difference likely reflects reduced proliferation rather than death, as TUNEL labeling revealed no differences between the double mutant and heterozygote at these stages (data not shown).

By E16.5, although ventricular expression of GFP in homozygotes persisted (Figure 6E, arrow), GFP^{+} cells could be seen migrating into the gray matter (Figure 6E, arrowheads). However, these cells took a more ventral trajectory than in heterozygotes. At E18.5, many GFP^{+} cells could be detected at the pial surface of the ventral white matter in homozygotes (Figure 6F, open arrowheads), a location not occupied by GFP^{+} cells in hetero-

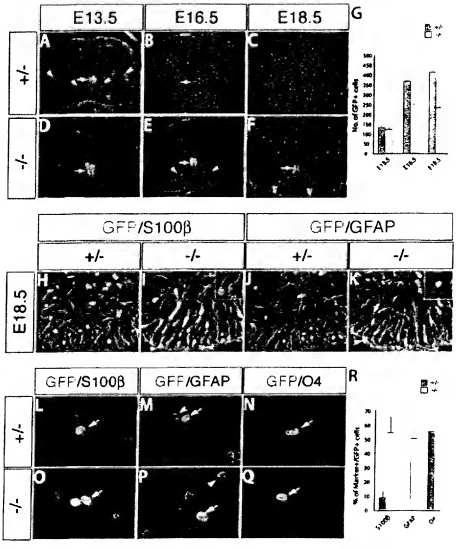

Figure 6. Oligodendrocytes Are Transformed into Astrocytes in the Absence of *Olig1* and *Olig2*

(A–G) Crossections of heterozygous and homozygous thoracic spinal cord at indicated stages were labeled with an anti-GFP antibody. Arrows indicate ventricular expression of GFP. Persistence of GFP was apparent in both somatic (A, red arrowheads) and visceral (A, yellow arrowheads) motoneurons in the heterozygotes at E13.5, a time when the migration of GFP$^+$ oligodendrocyte precursors just started (A, white arrowheads). Open arrows in (F) indicate GFP$^+$ cells located within pial surface. Quantitative analysis (G; mean ± S.D., eight sections from two embryos) revealed a difference in the number of GFP$^+$ cells in the null mutants versus heterozygotes at E16.5 and E18.5, but not E13.5.

(H–K) Many GFP$^+$ nuclei are associated closely with S100β$^+$ or GFAP$^+$ fibers in the ventral spinal cord of homozygotes (I and K) but not of heterozygotes (H and J) at E18.5. Arrows point to GFP$^+$ cells in the pial surface of the null mutant spinal cord. Inserts in (I) and (K) show double-labeled cells at higher magnification.

(L–R) Staining of acutely dissociated spinal cord cells at E18.5 from either heterozygotes (L–N) or homozygotes (O–Q). Arrows point to GFP$^+$ cells. Arrowheads in (M) and (P) mark GFP$^-$ GFAP$^+$ cells. Quantitative analysis (R) was performed by counting all GFP$^+$ cells from six different preps derived from different animals. Each prep contained ~5000 cells.

zygotes (Figure 6C). This observation suggested that the GFP$^+$ cells might have been transformed into astrocytes. To address this possibility, crossections of E18.5 heterozygous or homozygous spinal cord were double labeled with antibodies to GFP and the astroglial markers GFAP or S100β. This analysis revealed that many GFP$^+$ cells in the null mutant spinal cord coexpressed GFAP or S100β (Figures 6I and 6K, arrowheads and insets). In contrast, no colocalization of GFP with either of these markers was observed in the heterozygous spinal cord (Figures 6H and 6J), consistent with previous reports that *Olig2* is not expressed in cells of the astroglial lineage in wild-type embryos (Lu et al., 2000; Zhou et al., 2000).

The filamentous staining pattern of GFAP and S100β precluded an accurate quantification of the percentage of GFP$^+$ cells that were GFAP$^+$ or S100β$^+$ in vivo. To circumvent this problem, we performed double-labeling on acutely dissociated spinal cord cells from E18.5 heterozygous and homozygous embryos. Over 50% of GFP$^+$ cells in homozygous spinal cord coexpressed GFAP (Figures 6P, arrow, and 6R, GFAP, white bar). In sharp contrast, none of the GFP$^+$ cells in the heterozygous spinal cord was found to be GFAP$^+$ (Figures 6M, arrow versus arrowhead, and 6R, gray bars). Similarly, 44% to 66% of GFP$^+$ cells were S100β$^+$ in the homozygote (Figures 6O, arrow, and 6R, white bar), whereas less than 10% of GFP$^+$ cells in the heterozygote were S100β$^+$ (Figures 6L, arrow, and 6R, gray bar). Conversely, the oligodendrocyte cell-surface marker O4 decorated over 55% of GFP$^+$ cells in the heterozygous spinal cord (Figures 6N, arrow, and 6R, gray bar) but none in the homozygote (Figures 6Q, arrow, and 6R, white bar). The reciprocal change in the percentage of GFP$^+$ cells expressing O4 versus GFAP or S100β in heterozygous versus double homozygous embryos (Figure 6R) strongly suggests that in the absence of *Olig1/2* function, the *Olig2*-expressing cell population produces astrocytes rather than oligodendrocytes. Consistent with this conclusion, in cultures of neural progenitors from *Olig1/2* homozygous embryonic spinal cord, many Olig2-hGFP-expressing cells differentiated to astrocytes but none differentiated to oligodendrocytes, whereas in cultures from heterozygotes, many GFP$^+$ oligodendrocytes developed (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/109/1/61/DC1).

Discussion

The bHLH transcription factors OLIG1 and OLIG2 are sequentially expressed in motoneuron progenitors (Takebayashi et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001) and oligodendrocyte precursors (Lu et al., 2000; Zhou et al., 2000). Here we show that in the absence of *Olig1* and *2* function, motoneurons are converted to V2 interneurons in the spinal cord, while oligodendrocytes fail to differentiate throughout the nervous system. Our results suggest that oligodendrocyte precursors are not simply eliminated, but instead differentiate to astrocytes. These observations are consistent with the idea that in *Olig1/2* double mutants, *Olig2*-expressing progenitors sequentially generate interneurons and astrocytes rather than motoneurons and oligodendrocytes. In this way, *Olig* genes link the specification of a particular neuronal subtype to that of a specific glial subtype, independent of the decision between neuronal versus glial fates.

Olig2 Is Required for Both the Regional Identity and Differentiation of Motoneuron Precursors

Misexpression studies in the chick have suggested that OLIG2 plays two roles in motoneuron fate determination: it specifies the regional identity of the pMN domain via repression of *Irx3*, and it promotes motoneuron progenitor cell cycle exit and differentiation, in part via local derepression of *Ngn2* (Mizuguchi et al., 2001; Novitch et al., 2001). The loss-of-function data presented in this and the companion paper (Lu et al., 2002 [this issue of *Cell*]) strengthen this view. Combined deletion of *Olig2* and *Olig1* causes a derepression of *Irx3* in pMN and a loss of *Ngn2* expression in this domain. The selective loss of *Ngn2* expression in pMN is consistent with the idea that this bHLH factor is controlled by distinct *trans*-acting factors in different progenitor domains (Scardigli et al., 2001). The motoneuron deficit in the *Olig1/2* double knockout is similar to that seen in embryos lacking *Nkx6.1/6.2* (Vallstedt et al., 2001), a homeodomain patterning molecule (Briscoe et al., 2000) that is required for *Olig2* expression (Novitch et al., 2001). The fact that expression of *Nkx6.1* and *6.2* is unperturbed in *Olig1/2* knockouts is consistent with the idea that *Olig* genes function downstream of *Nkx6.1/6.2* in motoneuron generation (Novitch et al., 2001).

Olig Genes Function Cell Autonomously in Oligodendrocyte Fate Specification

The complete failure of oligodendrocyte formation in *Olig1/2* double mutants suggests that all oligodendrocytes require *Olig* genes. Consistent with this, oligodendrocytes are not generated in neurosphere cultures derived from *Olig1*$^{-/-}$*2*$^{-/-}$ spinal cord (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/109/1/61/DC1). The fact that *Olig1/2* are coexpressed in oligodendrocyte precursors (this study; Lu et al., 2000; Zhou et al., 2000) suggests that this defect likely reflects a cell-autonomous requirement for these genes. An alternative explanation, however, is that this phenotype is a non-cell-autonomous consequence of the earlier loss of motoneurons, which have been hypothesized to send a feedback signal to the ventricular zone to regulate the subsequent production of oligodendrocytes (Hardy, 1997; discussed in Richardson et al., 2000).

We think this hypothesis is unlikely, however, because in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ double mutants, neuronal differentiation in the ventral spinal cord is largely eliminated (Scardigli et al., 2001), but oligodendrocyte precursor formation is unaffected (our unpublished observations). Similarly, in *Isl1*$^{-/-}$ mice which lack motoneurons (Pfaff et al., 1996), oligodendrocyte differentiation is also unaffected (Sun et al., 1998). Finally, oligodendrocytes develop normally in the hindbrain of *Olig2* single mutants (Lu et al., 2002 [this issue of *Cell*]), which lack somatic motoneurons. Our observation that in *Olig1/2* double mutants, hindbrain oligodendrocytes are completely lost further indicates that the sparing of hindbrain oligodendrocytes in *Olig2*$^{-/-}$ embryos is not due to compensation of a somatic motoneuron-derived signal by visceral motoneurons, which are spared in both *Olig2*$^{-/-}$ and *Olig1*$^{-/-}$*2*$^{-/-}$ mutants.

Role of *Olig* Genes in Motoneuron and Oligodendrocyte Fate Specification

The inference that *Olig* genes function cell autonomously in oligodendrocyte development leaves open the question of when that function is required. Our data indicate that both motoneurons and oligodendrocytes are normally generated from pMN (but not from p2) and support the idea (Richardson et al., 1997, 2000) that these neurons and glia share a common precursor. Consequently, the homeotic-like transformation of such pre-

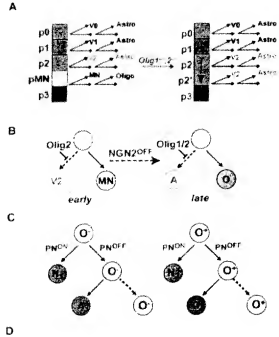

Figure 7. *Olig* Genes Control the Subtype Identities of Both Neurons and Glia Derived from a Common Progenitor Domain

(A) Summary of neuronal and glial phenotypes in *Olig1*$^{-/-}$; *Olig2*$^{-/-}$ mutants.

(B) *Olig* genes may act sequentially in motoneuron (MN) and oligodendrocyte (O) development. Abbreviation: A, astrocyte.

(C) Two putative types of spinal cord progenitor cells. O* progenitors do not express *Olig* genes and generate certain types of neurons (N_x) and later astrocytes (A). Abbreviations: PN, proneural. O* progenitors first generate other types of neurons (N_y) and then oligodendrocytes (O), blue cell.

(D) A simple combinatorial code composed of proneural and *Olig* genes can determine whether CNS progenitors produce neurons, oligodendrocytes, or astrocytes. $Neuron_x$ and $Neuron_y$ denote two different neuronal subtypes.

cursors from a pMN to a p2 identity in *Olig1/2* homozygous embryos could result in the elimination of both cell types (Figure 7A). In that case, the wild-type function of *Olig* genes in specifying both the motoneuron and oligodendrocyte fates might simply be repression of *Irx3* in pMN. Alternatively, the mutant phenotype could reflect an independent and sequential requirement for *Olig* genes in both early patterning of the pMN domain and in oligodendrocyte fate determination (Figure 7B).

Consistent with the idea of independent functions, the hindbrain phenotype of *Olig2*$^{-/-}$ single mutants demonstrates that *Olig* gene mutations can cause deficiencies in somatic motoneuron generation without necessarily affecting oligodendrocyte development (Lu et al., 2002 [this issue of *Cell*]). Furthermore, while misexpression of *Olig2* in chick is sufficient to cause ectopic repression of *Irx3* and motoneuron induction in some regions of the spinal cord (Novitch et al., 2001), it does not induce ectopic oligodendrocyte differentiation (Zhou et al., 2001). Thus, while repression of *Irx3* by *Olig* genes may be necessary for oligodendrocyte fate determination, it may not be sufficient. Formal resolution of this issue will require selective rescue of the early pMN phase of *Olig2* expression in *Olig1/2* double knockouts to determine whether both motoneurons and oligodendrocytes are recovered.

OLIG1 and 2 Control an Oligodendrocyte versus Astrocyte Fate Choice

Our lineage-tracing data suggest that many *Olig2-hGFP*-expressing precursors generate astrocytes instead of oligodendrocytes upon deletion of *Olig1* and *Olig2*. If so, it would imply that spinal cord oligodendrocyte precursors have the potential to generate astrocytes, but that this fate is normally repressed by *Olig* genes (Figure 7B, right). Astrocytes are thought to arise from multiple levels along the dorso-ventral axis of the spinal cord (Pringle et al., 1998). Thus, it is a reasonable assumption that the p2 domain normally generates astrocytes after it generates V2 interneurons (Figure 7A, left). If so, then the trans-fating of *Olig2*-expressing progenitors to astrocytes in *Olig1/2* double mutants could reflect a conversion of progenitors that sequentially generate motoneurons and oligodendrocytes to ones that produce first V2 interneurons and then astrocytes (Figure 7A, right, p2').

If deletion of *Olig* genes causes oligodendrocyte precursors to generate astrocytes, do such precursors normally generate astrocytes following downregulation of *Olig* gene expression in the ventricular zone? We found no evidence for persistence of the OLIG2-hGFP lineage marker into astrocytes in *Olig1/2* heterozygotes. Similarly, using a permanent lineage tracer, Lu et al. (2002 [this issue of *Cell*]) found no astrocytes among the progeny of *Olig1*-expressing cells. These data suggest that *Olig*-expressing progenitors normally produce motoneurons and oligodendrocytes but not astrocytes in vivo (Figure 7C, O*). This idea may seem inconsistent with the demonstration that single CNS progenitors can generate neurons, astrocytes, and oligodendrocytes in culture (reviewed in Gage, 2000; Anderson, 2001). However, despite extensive retroviral lineage tracing studies, there is no clear evidence for tripotential neuron/astrocyte/oligodendrocyte progenitors in vivo (Luskin et al., 1988; Leber et al., 1990). The tripotential CNS stem cells characterized in vitro may thus represent a more primitive progenitor than has been identified in vivo. Alternatively, the cell culture environment may reveal a combination of developmental potentials that are not actually used by any single progenitor in vivo.

The Genetic Logic of Neural Cell Fate Determination

bHLH proneural genes such as the *Ngns* control a neuron versus glial fate switch (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001b; reviewed in Vetter, 2001). In

the case of motoneurons and oligodendrocytes, *Ngn1/2* is likely to be the proneural gene that controls this switch (this study; Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). These data, taken together with our previous results (Zhou et al., 2001), suggest that once expression of *Ngns* has been extinguished in pMN, *Olig* genes determine whether the remaining progenitors will produce oligodendrocytes or astrocytes (Figure 7B).

These results suggest a simple combinatorial code whereby different combinations of the *Olig* and proneural genes can specify either neuronal, oligodendroglial, or astroglial fates (Figure 7D). According to this genetic logic, the astroglial fate would represent a final "ground state," in which neither proneural nor *Olig* genes are expressed. This fate may, however, require active repression by *Hes* genes, which repress proneural gene expression (Ishibashi et al., 1995; Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001; reviewed in Fisher and Caudy, 1998).

Combinatorial codes of Lim HD and Ets domain transcription factors have been shown to control different aspects of motoneuron subtype identity (Tsuchida et al., 1994; Lin et al., 1998; Sharma et al., 1998; Kania et al., 2000). By contrast, bHLH factors have until now been viewed as primarily acting in linear cascades to produce a single cell type, such as muscle or neuron (Weintraub, 1993; Lee, 1997). The results presented here suggest that in the nervous system, bHLH factors can also function in a combinatorial code that determines the three fundamental cell types of the CNS. This code may provide a foundation upon which higher-order aspects of neuronal and glial subtype identity can be built by superimposing combinatorial codes composed of other families of transcription factors. The linking of these multiple coding systems may then be achieved by crossregulatory, and perhaps physical, interactions between the molecules that comprise them.

Experimental Procedures

Generation of Olig1 and Olig2 Double Mutant Mice

All mouse genomic clones were derived from a 129/SVJ genomic library (Stratagene). Both the *Olig1* and *Olig2* sequences are encoded by a single exon. The *Olig2* targeting vector was constructed by inserting a *Histone-GFP fusion/loxp/PGKneo* cassette between a 2 kb 5′ arm and a 5.6 kb 3′ arm. For the *Olig1* targeting vector, a *tau LacZ/loxp/PGKhyg* cassette was cloned between the 1.6 kb 5′ arm and the 3.2 kb 3′ arm.

Two rounds of electroporation and selection were conducted, first with the *Olig2* targeting vector, and then with the *Olig1* targeting vector. Correctly recombined clones at both the *Olig2* and *Olig1* loci were subjected to Cre/Lox analyses to determine whether the two recombined alleles reside on the same chromosome (protocol available upon request). The frequency of recombination for both the *Olig2* and *Olig1* loci was ~1:300. Clones in which the two mutant alleles are located in *cis* were injected into C57BL/6J blastocysts to generate germline chimeric founders. Mutant mice were genotyped with PCR primers specific to *Olig1*, *Olig2*, *GFP*, *lacZ*, *Neomycin*, and *Hygromycin* genes. No segregation of the two mutant alleles has been observed in all embryos genotyped so far. All embryos analyzed in this study were derived from heterozygous 129sv × C57BL/6J intercrosses.

In Situ Hybridization

Nonradioactive in situ hybridization was performed as previously described (Zhou et al., 2000). The following mouse gene probes were used: *Olig1*, *Olig2*, *Olig3*, *Scx10* (a gift of Dr. Kirsten Kuhlbrodt), *PDGFRα*, *MBP*, *PLP/DM20*, *Ngn1*, and *Ngn3*. Probes for *Nkx6.1*, *Nkx6.2*, and *Dbx2* were the kind gift of Dr. Thomas Jessell.

Immunohistochemistry

Mouse embryos were fixed by immersion in 4% paraformaldehyde from 1 hr to overnight at 4°C depending on the age. The following primary antibodies were used: rabbit anti-Olig2 (1:2000, gift of Dr. Takebayashi Hirohide), rabbit anti-Nkx2.2 (1:1000, gift of Dr. Thomas Jessell), mAb anti-Neurogenin2 (1:100, Lecheng Lo), rabbit anti-Chx10 (1:4000, gift of Dr. Thomas Jessell), guinea pig anti-Irx3 (1:1000, gift of Dr. Thomas Jessell), rabbit anti-Hb9 (1:2000, gift of Dr. Samuel Pfaff), rabbit anti-GFP and Alexa 488 conjugated rabbit anti-GFP (1:1000, Molecular Probes), rabbit anti β-gal (1:1000, 5′-3′), rabbit anti-GFAP (1:1000, DAKO), mAb anti-S100 β (1:1000, Sigma), and rabbit anti-Phox2b (1:700, gift of Dr. Jean-Francois Brunet). mAbs against Lim3, MNR2/Hb9, Engrailed-1, Isl1/2, Hb9, Lim3, Nkx2.2, and Pax6 were obtained from Developmental Studies Hybridoma Bank (DSHB). Whole-mount antibody staining of mouse embryos was performed as described previously (Ma et al., 1996).

BrdU Labeling and TUNEL Assay

BrdU labeling of mouse embryos was conducted by intraperitoneal injection of BrdU (Sigma, 65 mg/g body weight) 2 hr before sacrifice. A rat anti-BrdU antibody (Accurate) was used to detect BrdU. TUNEL assays were performed with a kit from Roche according to the manufacturer's instructions.

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